

**METHANOL UTILIZING BACTERIA FOR
SINGLE-CELL PROTEIN PRODUCTION**

Lena Häggström

1975

**Chemical Centre
Technical Microbiology
University of Lund**

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SINGLE-CELL PROTEIN PRODUCTION

av

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Akademisk avhandling som för avläggande av filosofie
doktorsexamen vid tekniska fakulteten vid universitet
i Lund kommer att offentligen försvaras å Kemicentrum,
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Technical Center
Technical Microbiology
University of Lund
Lund, Sweden

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PREFACE

The present investigation is part of a larger research project on single-cell protein from methanol. Most of this work has been performed at the Dept. of Applied Microbiology, Karolinska Institutet, but since July 1974 continued at Technical Microbiology, Chemical Centre, University of Lund.

I wish to thank Prof. Nils Molin for his enthusiasm and support and the patience he has showed when results did not appear very fast. His way of creating a humane and stimulating working atmosphere has meant much to me. I am indebted to him, and to Dr. Milan Dostálek who introduced me to the field of continuous culture. All the indispensable pieces of advice he has given me in the daily work, have greatly contributed to the realization of this thesis. I also want to thank Prof. Carl-Göran Hedén for initiating the project on single-cell protein.

Mrs. Inger Hermansson made most of the experiments with great care and scilfulness and Miss Eva Melander helped me to finish the work. I am grateful to all my colleagues for their support in my work. Miss Anna Paulsson and Mrs. Anette Ahnell have typed the manuscript. My mother has corrected my English.

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Section I is based on the publication 'Studies on methanol oxidizing bacteria' Biotechnol. Bioeng. XI, 1043-54 (1969).

The electron microscopic investigation in section III was performed in co-operation with Dr. Bo Forslind, Dept. of Medical Physics, Karolinska Institutet and published separately 'Characteristics of Methylomonas methanolica: A preliminary study with the electron microscope' J. Ultrastr. Res. 44, 448-49 (1973)

Section IV was performed in co-operation with Prof. Nils Molin and Dr. Milan Dostálek and is based on the publication 'Optimization of biomass production from methanol' Proc. IV IFS: Ferment. Technol. Today 497-501 (1972)

INTRODUCTION

Single-cell protein (SCP) is protein in form of biomass from all kinds of microorganisms. There are several reasons why microorganisms are suitable for mass production of protein. They grow on all kinds of substrates and they grow fast if compared to plants and animals. The protein content in the dry biomass is high, between 40-60%. Microorganisms can be cultivated in large scale during controlled conditions and are easy to manipulate genetically. The main problems with this new type of protein are that long-term toxicological tests are necessary to avoid unexpected toxic effects and the unacceptably high content of ribonucleic acids. For these reasons SCP is so far primarily considered as a supplement to animal fodder.

Algae, bacteria, yeasts, and mycelial fungi have been used for SCP production. Each group has its own advantages and disadvantages, which does not mean that one excludes the other. Algae can utilize the readily available carbon dioxide in the air, but need on the other hand intensive light-energy. Bacteria have the highest growth rate of the four groups and a very high protein content, but are the most difficult organisms to separate from the growth medium. They have also the drawback of being an unconventional protein source. Yeasts have the indisputable advantage of having been used for food production in the history of mankind. Yeasts have also a relatively good cell composition but growth rate and protein content are lower than for bacteria. They are also relatively difficult to separate. Mycelial fungi has a broad enzyme spectrum, enabling them to attack a wide range of substrates. They grow on the other hand comparatively slowly and no other group of microorganisms are known to produce such a variety of toxic substances. Mycelial fungi are however very easy to separate and have been used for food processing since long.

The same argumentation applies also to the choice of substrate. There are no general terms in telling which substrate is the most advantageous one, or which substrate is less useful. If a SCP process is set up, it must be based on local conditions if being economically feasible. Principally exists two different processes for production of SCP. One is based on the utilization of industrial wastes such as all forms of carbohydrates e.g. molasses, sulfite liquor, starch, and cellulose. Such a process will at the same time contribute to the disposal of the wastes. The other type is based on petro products such as gas-oil, n-alkanes, methane, and alcohols.

If biomass production from bacteria is used as a basis for a comparison of glucose, methanol, methane, and higher n-alkanes (1), one will find that the substrate yield, calculated as g biomass produced per g substrate consumed, is highest for n-alkanes. Oxygen demand is lowest for growth on glucose followed by methanol, n-alkanes and last methane. Heat production follows the same pattern as oxygen consumption. Methanol as a substrate has doubtless advantages compared to other petro products. Bacteria and yeasts grow readily on it. Methanol is easily produced from all types of raw materials without the presence of a refinery. It is completely miscible with water, in contrary to hydrocarbons, and easy to remove from the product. Methane is otherwise considered as one of the most attractive carbon sources for SCP production due to the extremely low costs for the substrate. There are many instances today where the gas is not regained but simply consumed by burning at the oil fields. The problems encountered with methane are its explosive character in air-mixtures, the difficulties in getting enough methane dissolved in the fermentation broth, and the high heat evolution during microbial growth.

The present investigation concerns the production of SCP in form of bacterial biomass from methanol, with emphasis on the following concepts: 1) Isolation and selection of new strains

2) Characterization of strains 3) Optimization of cultivation conditions for the strain 4) Evaluation of strain-specific properties, relevant to SCP production 5) Strain improvement

In this investigation, three different cultures are used for studies on biomass production from methanol. These will be described in regard to their suitability for SCP production but also in physiological terms. An ideal production strain should be in possession of a number of relevant characters, such as a high growth rate and ability to grow during conditions that correspond to high productivity. The organism should utilize the carbon substrate efficiently and have a minimal oxygen requirement. Aeration in large scale cultivations is one of the great economical problems, and oxygen deficiency is the main reason for limitation of the biomass yield. Another aspect on the cellular oxygen demand is the heat evolution during growth. Heat evolution is in direct proportion to oxygen consumption (1). Cooling of big industrial fermenters requires enormous amounts of cooling water adding further economical load to the process. As a consequence of this, the ideal production strain should also have a high temperature optimum for growth, thereby diminishing cooling costs. The strain should further be resistant to extreme conditions, such as low pH and be easy to separate from the growth medium. Its nutritional value, as judged by the amino acid composition and by animal feed tests, must also be satisfactory.

When starting a project for developing a SCP process it naturally begins with the isolation of new strains from nature. The suitability of a certain strain cannot be assessed until the whole range of relevant characters have been investigated. This is a time-consuming and elaborate work. In practice, selection of a strain must be based on such characters which can be determined relatively easy e.g. growth rate and biomass yield in batch culture. The possibility to find the ideal strain from the beginning is thus not very likely. Whether the selected strain is perfect or

not, it is of great importance to find optimal cultivation conditions. As will be seen in this report much can be gained by adjusting the environment in which the microbe is growing.

Strain improvement is equally important in SCP processes as in other microbial fermentation processes. Experiences of strain improvement from fields like antibiotic production show clearly that these processes would otherwise not have been economically feasible today. A part of this investigation is also devoted to this concept. All these properties which characterize an ideal production strain are of course the subjects for improvements. In this investigation attempts have been made to increase the temperature tolerance in a methanol utilizing bacterial strain. Most methanol utilizing bacteria reported in the literature have temperature optimum around 30°C, but there are exceptions, such as the mixed culture of methanol utilizing bacteria isolated by Snedecor and Cooney (2) which grow well at 50-56°C.

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GENERAL METHODS

All methods used in more than one of the individual sections will be described under this heading. The methods used in the individual sections are then listed under the heading "Materials and Methods" using the same nomenclature as below. If a special method has been applied, it will be described in the appropriate place.

Cultures

Three cultures of methanol utilizing bacteria have been used in this investigation.

1. A mixed culture named TM 20.
2. A pure strain named Methylomonas methanolica.
3. A mutant strain named M13V, derived from M. methanolica.

Storing conditions for cultures

Stock cultures of all three strains were kept lyophilized. New ampoules were opened when necessary. The cultures were then routinely kept on agar media at +4°C and reinoculated approximately once every two weeks, except for culture TM 20 (see section I).

Inoculum preparation

Inoculum for all types of experiments was prepared in at least two serial liquid shaken cultures. Colonies from agar plates were transferred to 25 ml of growth medium in a 100 ml conical flask and incubated at the appropriate temperature. The cells were centrifuged and transferred to 100 ml growth medium in a 300 ml flask. Before used in an experiment the inoculum was always centrifuged and resuspended in the MM2 salts solution (see below). If more cells were needed, additional transfers in increasing volumes were made. Inoculum size for shake culture experiments was in the order of 0.01 g dry weight of cells·l⁻¹ and for fermenter cultivations 0.03 g dry weight of cells·l⁻¹.

Media

1. MM2 salts solution contained per litre aq. dest.:

NH_4Cl	0.8	g
Na_2HPO_4	0.6	g
KH_2PO_4	0.4	g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	0.2	g
$\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$	8.35	mg
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$	0.33	mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.09	mg
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.09	mg
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.08	mg
$\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$	0.08	mg

The pH of this solution is 6.7

2. MM2 growth medium is the MM2 salts solution supplemented with 0.3% (v/v) methanol.

3. MM2 agar medium. Solid plates were prepared by adding 1.5% (w/v) Noble agar (Difco) to MM2 salts solution. After autoclaving and cooling to 45°C , 0.5% (v/v) methanol was added to the medium.

4. W8 + W9 are the mineral media developed in section IV. W8 is the growth solution and W9 is a dosing solution. The relative composition of these are identical but W9 is more concentrated. The media are prepared in a concentrated form described below. The concentration during growth conditions is characterized by the phosphorus content. Cultivations were routinely carried out at $350 \text{ mg P} \cdot \text{l}^{-1}$.

Concentrated W8 and W9 containing $10 \text{ g P} \cdot \text{l}^{-1}$ has the following composition per litre aq. dest.:

H_3PO_4	26.16	g
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$	8.10	g
H_2SO_4	7.04	g
$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	3.76	g
KH_2PO_4	3.48	g
$\text{Fe}(\text{SO}_4)_3 \cdot 7\text{H}_2\text{O}$	1.88	g
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0.14	g
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	86.00	mg
$\text{MnSO}_4 \cdot \text{H}_2\text{O}$	12.32	mg
H_3BO_3	2.28	mg
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	1.60	mg
Na_2MoO_4	0.84	mg

In order to avoid precipitation 5.71 g citric acid was added per litre aq. dest.

The growth solution containing $350 \text{ mg P} \cdot \text{l}^{-1}$ has a pH of 2.1. By titration with NH_4OH , pH was adjusted to the desired growth condition, adding nitrogen to the medium at the same time. Adjusting pH to 6.0 corresponds to a nitrogen content of $0.4 \text{ g} \cdot \text{l}^{-1}$.

During growth W9 was dosed to the culture at the same rate as the ammonia solution used for pH control. For balanced cultivation conditions as described in section IV, the ammonia solution contained $20 \text{ g N} \cdot \text{l}^{-1}$ and W9 contained $5 \text{ g P} \cdot \text{l}^{-1}$.

Cultivation conditions

1. Shaking flasks were incubated in a thermostated shaking table with rotatory movements. If not otherwise stated routine incubation temperature for culture TM 20 and M. methanolica was 30°C , and for strain M13V 37°C . The MM2 growth medium was always used.

2. Batch cultures in fermenters were performed either in a Biotec FL 103 fermenter, equipped with different fermenter walls so the working volume could be varied from 2-4 l or in a Chemoferm fermenter with a working volume of 4 l. pH and temperature were automatically controlled. All fermenter cultivations were performed at pH 6.0 using NH_4OH for adjusting the pH. Culture TM 20 and M. methanolica were grown at 30°C. For strain M13V see sections V, VI, and VII.

The air flow to the fermenter was kept constant by a pressure regulator and a flow meter and passed a sterile filter before inlet in the fermenter. Outgoing gas passed a condenser for minimizing of evaporation, a heater, and a final sterile filter before let out.

The fermenters were equipped with magnetic coupling to the 6 blade open turbine impellers and stepless motor speed control. Maximum oxygen transfer rate (OTR), as measured by the sulfite oxidation method, was approximately $400 \text{ m mol O}_2 \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ at a stirring speed of 1 700 rpm and an aeration rate of 2 VVM (1). This was the highest OTR which could be obtained in practice and is referred to as standard cultivation conditions in the text. If lower OTR values were used this will be stated if of any significance.

Dissolved oxygen in the growth medium was measured by a probe according to Johnson et al. (3). 100% dissolved oxygen is the saturation value of the liquid in equilibrium with air.

The methanol concentration in the medium was measured and automatically controlled by using a catalytic combustion analyzer (Alcotrol Ac 3, Servo-Chem AB). A concentration of 0.3% (v/v) methanol was routinely maintained in batch cultures.

The amounts of dosed ammonia solution and dosed methanol were continuously measured by two dose monitors (DM1, Servo-Chem AB).

Values on dissolved oxygen, pH, methanol concentration, amount of dosed ammonia, and amount of dosed methanol were continuously recorded by a multipoint recorder.

3. Continuous culture was accomplished by using the same basic equipment as for batch culture. Medium inflow was regulated by a membrane pump (Fe 211 B. Braun-Melsungen AG). When chemostat principles were applied, using methanol as the limiting substrate, the carbon source was supplied with the inflow medium. Cultivation volume was kept constant by a conductivity type level regulator connected to a peristaltic pump. Steady state was checked by the stability in optical density, dissolved oxygen and dry weight of cells.

Sterility

Appropriate sterile conditions were provided for all types of cultivations. Media, cultivation vessels, and containers for dosing solutions were autoclaved at 121°C for the time required. Ammonia solutions were prepared from sterile water and methanol was membrane filtered.

Analyses

Optical density (OD) was measured either in a Linson 3 colorimeter or in a Turner colorimeter at 620 nm. Appropriate dilutions were made when required. The Turner instrument gave approximately an OD value twice that of the Linson instrument. It will be stated in each section which of the two instruments that was used.

Bacterial dry weight was measured in two ways. For culture TM 20 and strain M13V, 10 ml samples were centrifuged at 3 200 x g for 30 min. in predried centrifuge tubes. The supernatant was drained off and the tubes allowed to dry in 105°C over night. For M. methanolica, dry weight was measured by filtering 10 ml samples (or less if the culture was too dense) through 0.45 µm Millipore membrane filters and drying the filters for 2 hours at 105°C. In both cases the samples were cooled over silica gel and thereafter weighed. Determinations were always made in at least duplicate.

Methanol concentration in growth medium or in flow medium for continuous culture was measured gas chromatographically. Samples from growth media were membrane filtered and analysed immediately or stored frozen. 1 μ l samples were injected in a Varian model 940 with a 6' x 1/8" Porapak Q column at 130°C and flame ionization detector.

Metals in biomass and in media were analysed by atomic absorption. Media samples were membrane filtered and acidified with concentrated HCl (0.5 ml/100 ml) and then diluted as required. Cells were centrifuged at 2 000 x g for 30 min., the supernatant was drained off and the cells freeze-dried. The dry material was then dissolved in boiling concentrated HNO₃ added with a few drops of 30% H₂O₂. After digestion the samples were cooled, diluted, and filtered for removal of such lipids that were not destroyed by this treatment. The samples were analysed in a Varian Techtron model AA-5 Atomic Absorption spectrophotometer according to standard procedures. Air-acetylene flame was used for all metals, except for Ca and Mo for which a nitrous oxide-acetylene flame was used. CsCl was added to samples analysed for K and Ca to avoid ionization effects.

Phosphorus was estimated as phosphate in the same samples as above according to Fiske and Subbarow (2).

Carbon, nitrogen, and hydrogen in biomass (freeze dried samples) were analysed in a Carlo Erba model 1102 Automatic Elemental Analyzer according to standard procedures (Carlo Erba Instrumentation, P.O.Box 4342, Milan, Italy).

Ammonium nitrogen in media samples was determined after addition of alkali and steam distillation of the ammonia into 3% boric acid. The absorbed ammonia was determined by automatic titration with 0.01 M HCl to pH 5.2.

Amino acids in biomass were analysed at the Amino Acid Analysis Laboratory, Dept. of Biochemistry, University of Uppsala. Analyses were performed after conventional acid hydrolysis.

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I. BIOMASS PRODUCTION FROM METHANOL USING A MIXED BACTERIAL CULTURE

SUMMARY

A mixed culture of methanol utilizing bacteria (TM 20) was cultivated on an inorganic salts medium supplemented with methanol as the sole source of carbon and energy. Biomass production was studied at different cultivation conditions. Maximum values from batch culture in a laboratory fermenter were: Specific growth rate 0.38 h^{-1} , productivity $0.90 \text{ g} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$, and biomass yield $11.0 \text{ g} \cdot \text{l}^{-1}$. The yield coefficient for methanol was estimated to $0.34 \text{ g} \cdot \text{g}^{-1}$, the methanol carbon conversion factor to $0.41 \text{ g} \cdot \text{g}^{-1}$, and the nitrogen conversion factor to $0.73 \text{ g} \cdot \text{g}^{-1}$. Crude protein in the biomass was 71.3%, amino acid protein 61.8%, and essential amino acids 24.5%.

INTRODUCTION

During the initial studies on biomass production from methanol, a mixed bacterial culture was used. This culture was originally isolated from soil, for growth on methane as the sole source of carbon and energy (1). If the culture was transferred to a mineral medium supplemented with methanol, it readily adapted to these conditions. After repeated subcultivations on methanol it lost, however, its capacity to utilize methane. This phenomenon depends probably on two factors. Methanol is extremely toxic or inhibitory to many methane utilizing bacteria (6). Other methane utilizers can grow on methanol as well (6), but it is possible that the growth rate on methanol is slower for these organisms than for organisms capable of using only methanol. An adequate comparison is though difficult to make because experimental data on growth rates, reported in the literature, are obtained during different cultivation conditions. After successive cultivations on methanol the methane utilizers in the mixed culture may thus be diluted out of the culture. The presence of methanol utilizing bacteria, not capable of utilizing methane in mixed methane cultures, have also been reported by others (7).

The mixed culture, adapted to growth on methanol was distinguished as culture TM 20 and investigated in order to know if it was suitable for SCP production. Growth characteristics in batch cultivations were examined. In the present section is also described a part of the development of proper cultivation conditions and control facilities for studies of biomass production in laboratory scale.

MATERIALS AND METHODS

Culture

The mixed culture TM 20 consists of Gram-negative small rods with an average size of $1 \times 0.5 \text{ }\mu\text{m}$. Plating to solid medium reveals differences in pigmentation and colony morphology.

Medium

MM2 growth medium for all types of cultivations.

Inoculum

Stock cultures of TM 20 were kept lyophilized. Agar media were never used in this purpose, because it would give rise to selection when the culture was transferred back to liquid medium. Inoculum was prepared as described in general methods but the first shaking flask was inoculated with a lyophilized sample.

Cultivation conditions

1. A Biotec FL 110 fermenter, with a cultivation volume of 10 l was used for batch cultures. Temperature and pH was automatically controlled. For pH regulation 0.5 M NaOH or NH_4OH was used. The driving unit had magnetic coupling to the impeller. Maximum stirring speed was 690 rpm. Aeration was controlled by a flow meter but without pressure regulation. Aeration rate was 0.3 VVM. OTR was less than $100 \text{ mmol O}_2 \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ during these conditions.

2. Biotec (2.5 l) fermentation unit equipped for batch culture as described in general methods. Aeration conditions correspond to an OTR of $300 \text{ mmol O}_2 \cdot \text{l}^{-1} \cdot \text{h}^{-1}$.

Analyses

Optical density estimations (Linson 3)

Bacterial dry weight

Methanol concentration in growth medium

Amino acids in biomass

Ammonium nitrogen in the medium was analyzed with a Technicon Autoanalyzer according to the method of Docherty (2)

Nitrogen and carbon in biomass were analyzed by Analytica AB, Sollentuna, Sweden.

RESULTS

Influence of pH, temperature and methanol concentration on growth rate

From a series of batch cultivations in 10 litres scale optimum pH, temperature, and methanol concentration were determined on basis of growth rate in the logarithmic phase. Optimum pH was 6.0-6.3. The culture was not too sensitive for changes in pH and could readily be grown between pH 5.5 and pH 7.0.

Temperature optimum was 30-31°C. The growth rate was reduced at 25°C and at 35°C. 40°C allowed slow growth but no growth occurred at 45°C. For maximum growth rate the methanol concentration should be kept below 0.5% (v/v). With more than 0.5% of methanol the growth rate was successively reduced.

Growth characteristics of culture TM 20 during different cultivation conditions

The first approach to the growth studies on culture TM 20 was batch cultivations in 10 litres scale. It was established that linear relationships existed between a) OD and biomass (Fig.1) b) OD and methanol consumption (Fig.2) c) OD and ammonium chloride consumption (Fig.3) d) OD and sodium hydroxide

dosed for pH control (Fig.4). These relations are characterized by the following equations (calculated by linear regression analysis):

$$a) \text{ Biomass } (g \cdot l^{-1}) = 0.77 \cdot OD + 0.07$$

$$b) CH_3OH_{\text{consumed}} (ml \cdot l^{-1}) = 3.08 \cdot OD + 0.39$$

$$c) NH_4Cl_{\text{consumed}} (g \cdot l^{-1}) = 0.46 \cdot OD + 0.04$$

$$d) NaOH_{\text{added}} (g \cdot l^{-1}) = 0.34 \cdot OD + 0.01$$

The biomass carbon content is 48% and the nitrogen content is 11.4%. From these data and the equations above the yield coefficient for methanol (g biomass produced/g methanol consumed), the methanol carbon conversion factor ($C_{\text{biomass}}/C_{\text{methanol}}$), and the ammonium nitrogen conversion factor ($N_{\text{biomass}}/N_{\text{ammonia}}$) could be calculated (Table 1).

In the early phase of this work methanol was added to the culture in portions, in order to keep the concentration at low levels for avoiding of toxic effects. The discovered relations between biomass production and consumption of methanol and dosed sodium hydroxide made it however possible to devise a mixture of sodium hydroxide and methanol which could be dosed during growth by the ordinary pH control unit. Methanol was thus dosed approximately in relation to the consumption rate, and an approximate constant level of methanol could be maintained. This system was not safe throughout since changes in the metabolism to higher or lower yield coefficient for methanol would lead to overdosing or to methanol limitation.

Biomass production in batch culture during these conditions is illustrated in Fig. 5. The maximum specific growth rate μ_{max} was 0.22 h^{-1} , biomass yield $1.4 \text{ g} \cdot l^{-1}$, and maximum productivity $0.17 \text{ g} \cdot l^{-1} \cdot \text{h}^{-1}$. The abrupt cease of growth was found to depend on nitrogen exhaustion of the medium. Exchanging the sodium hydroxide to ammonia for pH control resulted in a prolongation of the declining phase, increasing maximal productivity to $0.19 \text{ g} \cdot l^{-1} \cdot \text{h}^{-1}$ and final biomass yield to $4.5 \text{ g} \cdot l^{-1}$.

The need for proper equipment and control facilities became more pronounced as the work on biomass production continued. The instrumentation and control facilities for fermenter cultivations, as described in general methods, were successively worked out at the laboratory where this work was done.

By using a smaller cultivation volume, with possibilities to a complete control of the methanol concentration in the medium, and of the aeration rate and the level of dissolved oxygen in the fermenter substantially improved biomass production from the mixed culture. The growth rate increased to $\mu_{\max} = 0.38 \text{ h}^{-1}$, maximum productivity to $0.90 \text{ g} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$, and final biomass yield to $11 \text{ g} \cdot \text{l}^{-1}$ (Fig.5). All figures on biomass production from culture TM 20 are summarized in Table 1.

Table 1. Growth characteristics of culture TM 20 in batch culture during different cultivation conditions.

A = 10 l cultivation volume. pH controlled with sodium hydroxide mixed with methanol.

B = 10 l cultivation volume. pH controlled with ammonia mixed with methanol.

C = 2.5 l cultivation volume. pH controlled with ammonia. Methanol controlled separately.

	A	B	C
Maximum specific growth rate $\mu_{\max} \text{ (h}^{-1}\text{)}$	0.22	0.22	0.38
Biomass yield ($\text{g}\cdot\text{l}^{-1}$) at the end of the logarithmic phase	0.8	0.8	2.0
Final biomass yield ($\text{g}\cdot\text{l}^{-1}$)	1.4	4.5	11.0
Productivity ($\text{g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$) at the end of the logarithmic phase	0.16	0.16	0.43
Maximum productivity ($\text{g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$)	0.17	0.19	0.90
Yield coefficient for methanol ($\text{g}\cdot\text{g}^{-1}$)	0.32	0.32	0.34
Conversion factor for methanol carbon ($\text{g}\cdot\text{g}^{-1}$)	0.40	0.40	0.41
Conversion factor for ammonium nitrogen ($\text{g}\cdot\text{g}^{-1}$)	0.73	0.73	-

Protein content

The amino acid composition and protein content of culture TM 20 were analysed. The results are given in Table 2 and Table 3.

Table 2. Amino acid composition of culture TM 20.

Amino acid	g/100 g biomass	Amino acid	g/100 g biomass
Lysine	3.78	Arginine	5.06
Threonine	3.22	Cystine	0.23 ^a
Methionine	1.29 ^a	Tyrosine	2.07
Tryptophan	- ^a	Alanine	5.64
Isoleucine	2.78	Aspartic acid	6.03
Leucine	4.96	Glutamic acid	7.78
Valine	4.17	Glycine	3.95
Phenylalanine	2.98	Proline	2.71
Histidine	1.23	Serine	3.58

a) Methionine and cystine are partly destroyed by the acid hydrolysis and tryptophan is completely destroyed.

Table 3. Protein content of the mixed culture TM 20.

	g/100 g biomass
Total nitrogen (N_T)	11.4
Crude protein ($N_T \cdot 6.25$)	71.3
Amino acids according to analysis	60.5
Estimated losses during hydrolysis (tryptophan, cystine, methionine)	1.3
Total amino acids (N_{AT})	61.8
Essential amino acids	24.5
Non protein nitrogen ($N_T \cdot 6.25 - N_{AT}$)	9.5

DISCUSSION

The results reported here on biomass production from culture TM 20 show the importance of proper equipment and control facilities for microbial cultivations. During the more advanced cultivation conditions there was a tremendous increase in growth rate, productivity, and final biomass yield but the conversion

efficiency of methanol was not affected appreciately. The main reasons for these improvements were unsatisfactory control of the methanol concentration and oxygen limitation in the 10 litre cultivations.

Biomass production from mixed cultures have been reported both for methane utilization (1,3,5) and methanol utilization (4). In the early studies on biomass production from methane, it was considered that mixed cultures were superior to pure cultures in respect to growth rate (3,5). The mixed culture used here cannot compete with the pure strains described in the following sections, if one compares growth rate and productivity. The differences in carbon conversion efficiency are though less significant. The results presented here are, however, not directly comparable to the corresponding figures for the pure strains (section IV and VII) because these are cultivated during optimal conditions. It is quite possible that the final biomass yield and the productivity for culture TM 20 can be increased by adjusting the growth environment. The following sections will though show that batch cultivations alone cannot be used as the only method for evaluation of a culture aimed at SCP production.

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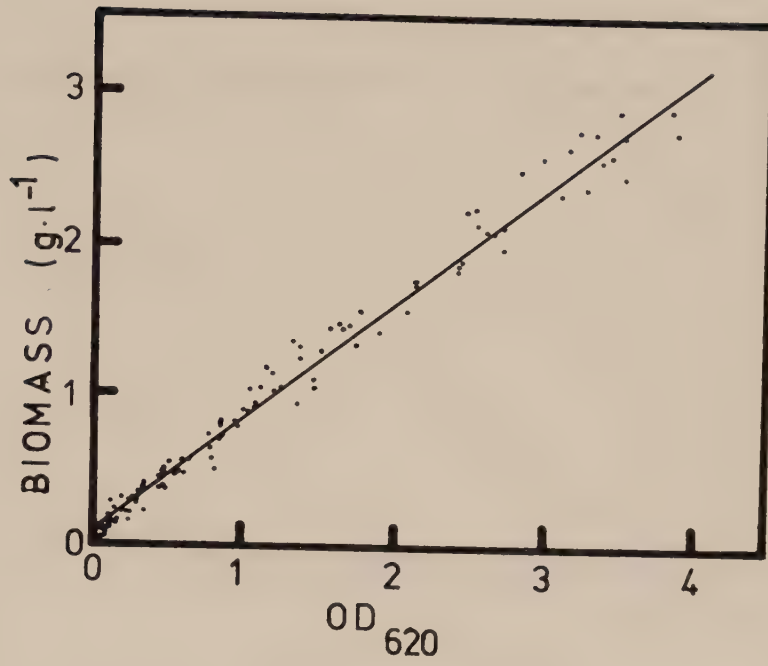


FIG 1. RELATION BETWEEN BIOMASS CONCENTRATION AND OPTICAL DENSITY (OD) IN BATCH CULTURES OF THE MIXED CULTURE TM 20.

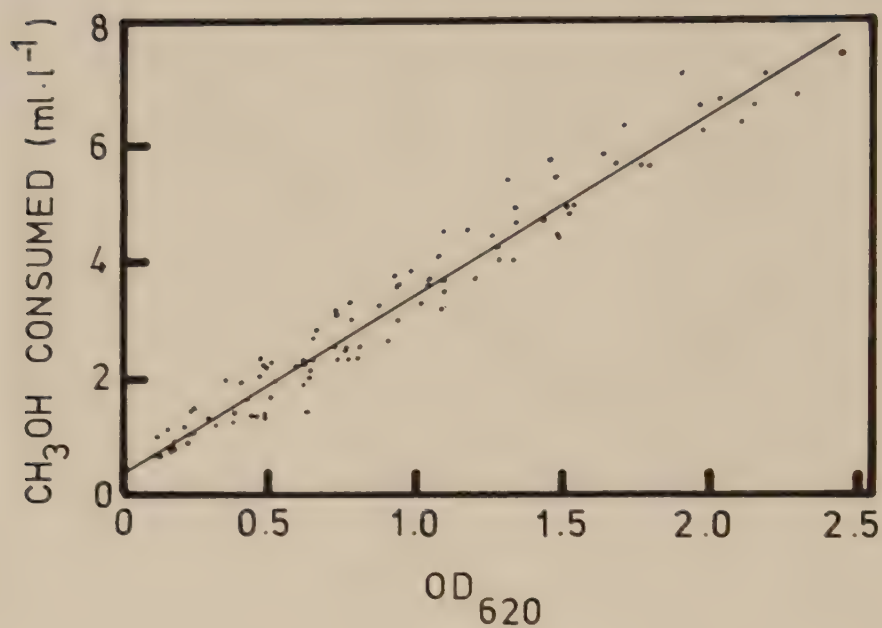


FIG 2. RELATION BETWEEN CONSUMPTION OF METHANOL AND OPTICAL DENSITY (OD) IN BATCH CULTURE OF THE MIXED CULTURE TM 20.

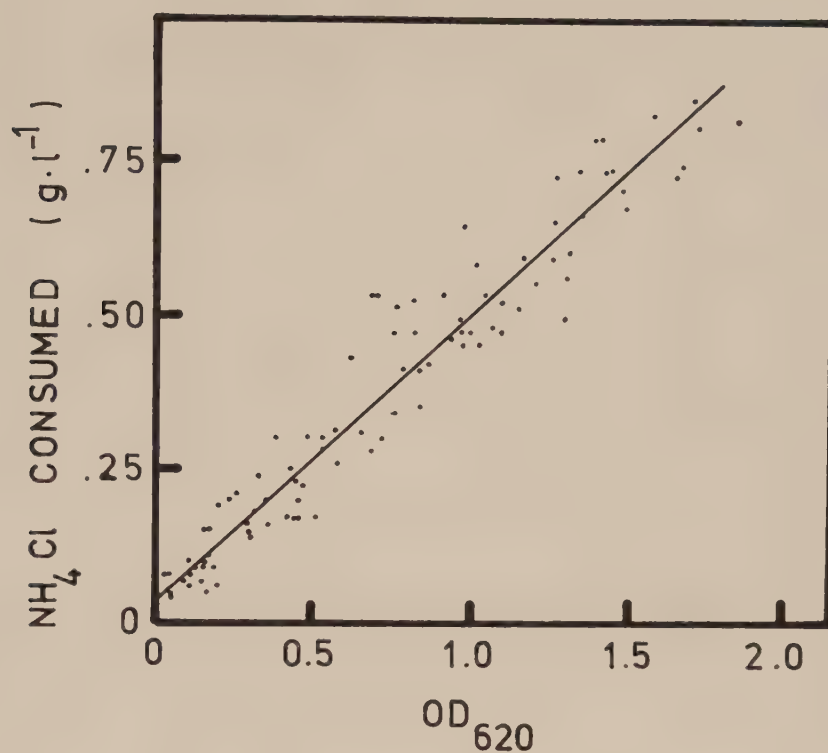


FIG 3. RELATION BETWEEN CONSUMPTION OF AMMONIUM CHLORIDE AND OPTICAL DENSITY (OD) IN BATCH CULTURE OF THE MIXED CULTURE TM 20.

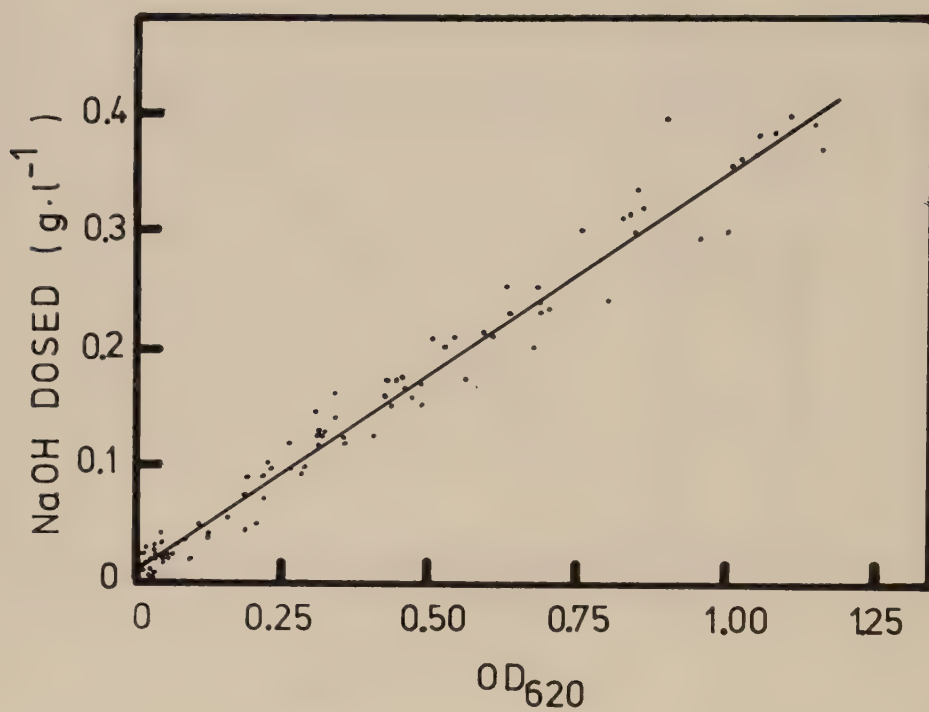


FIG 4. RELATION BETWEEN AMOUNTS OF DOSED SODIUM HYDROXIDE AND OPTICAL DENSITY (OD) IN BATCH CULTURE OF THE MIXED CULTURE TM 20.

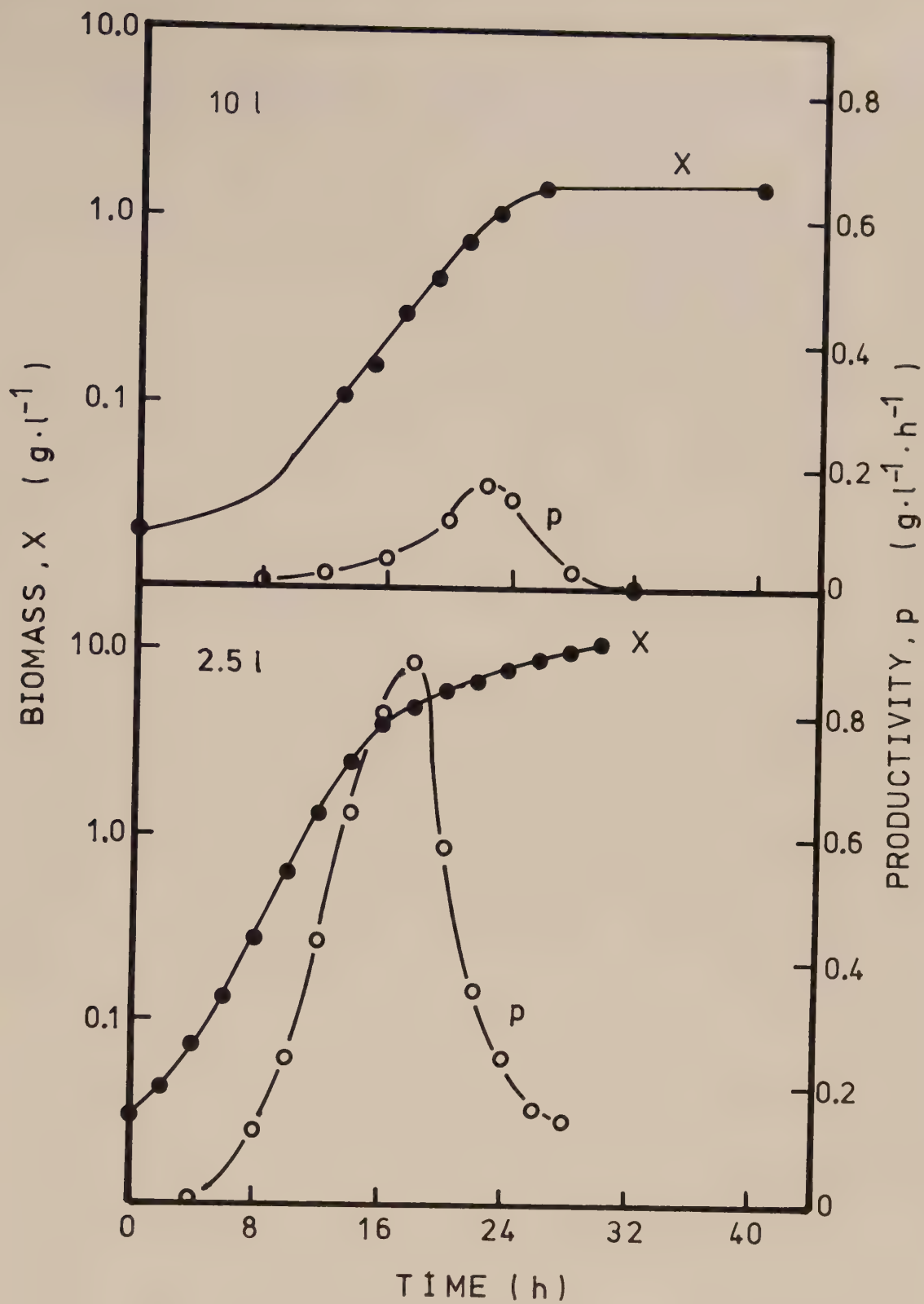


FIG 5. GROWTH AND PRODUCTIVITY FOR THE MIXED CULTURE TM 20. THE UPPER CURVE SHOWS A BATCH CULTURE IN 10 L SCALE AND THE LOWER CURVE A BATCH CULTURE IN 2.5 L SCALE.

11. ISOLATION OF PURE METHANOL UTILIZING BACTERIAL STRAINS

SUMMARY

The mixed culture TM 20 consists mainly of four individual strains. These were isolated in pure culture but none of the strains would reach the same growth rate as the mixed culture. A method for isolation of pure methanol utilizing bacterial strains from nature was worked out. A number of different species were isolated, one of which was chosen for the coming work on biomass production from methanol.

INTRODUCTION

The need for pure methanol utilizing strains became evident when the mixed culture TM 20 was grown in continuous culture. Prolonged cultivation apparently changed the composition of the culture. Attempts were thus made to study composition and stability in batch and continuous culture and isolation of the various strains in pure culture. As none of the pure strains reached the same growth rate as the mixed culture, a program for isolation of pure methanol utilizing bacterial strains from nature was carried on.

MATERIALS AND METHODS

Cultures

See the result section

Media

MM2 growth medium and MM2 agar medium.

Cultivation conditions

1. Shaking flasks 25-100 ml cultivation volume.
2. Batch cultures in Biotec (2.5 l) fermentation unit.
3. Continuous culture in Biotec (2.5 l) fermentation unit.

Principles for oxygen limited chemostat were applied.

Analyses

Optical density estimations (Linson 3)

Bacterial dry weight

RESULTS

Composition of culture TM 20

On agar plates from batch cultures of TM 20 four main colony types could be distinguished, which were isolated in pure form. Strain TM 21 forms pink pigmented colonies and is a rod-shaped, Gram-negative bacterium with one polar flagellum. The organism seemed to be similar to other pink pigmented Pseudomonas species reported in the literature capable of utilizing methanol (1,4,5,6). It also utilizes other carbon sources as well. TM 21 is the only separate strain in the mixed culture that grows satisfactory in liquid mineral medium with methanol, although growth is slower than in the mixed culture.

Strain TM 22 forms very small unpigmented colonies and is probably a Hyphomicrobium sp. The ovoid shaped Gram-negative cells are connected by thread like structures visible in the phase contrast microscope. At repeated transferences in liquid medium growth successively becomes slower, indicating that there is a requirement for growth factors. Doman et al. have earlier shown that H. vulgare is capable of utilizing methanol(3). Strains TM 23 and TM 24 form colonies on methanol agar plates, and can be kept there for a period of time, but none of the strains grow in liquid medium.

In addition to these strains 3-4 other types of organisms could always be detected on agar plates of the mixed culture, but in spite of repeated efforts it was impossible to obtain those

in pure culture. They apparently failed to form colonies when being isolated. This illustrates the complex interactions in the mixed culture. Some of the strains might need growth factors which they obtain in the mixed culture. Some of them might not even grow on methanol, but feed on lyse products or excreted compounds. It is even possible that one or more strains are subjected to phage attacks. Plaques were occasionally formed on plates with the mixed culture, but no attempts were made to investigate the circumstances around this matter. Strain TM 21 seemed, however, to be resistant, as a pink colony could develop in the middle of a clear zone.

The proportions between the different strains were constant both within one batch culture and at least in three serial batches. The culture is dominated by strain TM 22, which makes up 94% of the colony count. Strains TM 21, TM 23 and TM 24 are present in 2% each. The other strains that never were isolated in pure culture, constitute a considerably lower proportion of the culture.

The composition of culture TM 20 in an oxygen limited chemostat, inoculated with a lyophilized sample, was identical with the batch cultures in the initial period. A steady state could usually not be maintained for more than three days, whereafter the culture was washed out. At that time the colony pattern on agar plates had changed remarkably. Most conspicuous was the disappearance of strain TM 21 and the introduction of a new organism, not being observed earlier, which now completely dominated the whole culture. This change was invariably observed through repeated continuous cultures. In this context it should be pointed out that contamination control of mixed cultures is difficult. The new organism was however isolated in pure culture. It is capable of utilizing methanol, but occasionally fails to form colonies or grow in liquid medium. Whether this strain was a contaminant, or had been present in the culture from the beginning, is not clear.

Enrichment and isolation of methanol utilizing bacterial strains from nature

Samples from various sources (soil from mixed forest ground, water and mud from ponds, compost earth, sewage sludge) were used in enrichment cultures. A spoonful of the sample was inoculated into 100 ml liquid medium supplemented with 1% (v/v) methanol and incubated statically at 30°C for 4-7 days. Serial dilutions were made before spreading the enrichments on agar plates containing methanol as the sole carbon source. After an incubation period of 6-7 days, single colonies were picked, dispersed in fresh medium and spread on new plates. This procedure was repeated until a pure culture was obtained. The purity of the cultures were also checked by microscopic examinations.

The pure cultures were routinely tested in liquid medium to check their ability to utilize methanol as the sole source of carbon and energy. To be considered as a true methanol utilizer, a culture had to grow equally well in several serial shaken subcultures with a total dilution of 10^{-5} (after centrifugation one tenth of the culture was transferred to fresh medium of equal volume). The necessity of confirmative tests of presumptive methanol utilizers in several serial subcultures is illustrated by the following example: From one of the enrichment cultures a bacterial species was isolated in pure culture according to the description above. Methanol had, however, not been added to any of the media. The organism then grew in two liquid subcultures, still without any carbon source, before growth finally ceased.

In the initial phase of this work, different methods for enrichment and isolation were tried before the method described above was routinely used. This also turned out to be the simplest one.

In one series of experiments, methanol was added to the enrichment cultures in various concentrations from 0% to 20%. In cultures with more than 10% methanol very few bacteria developed, but different types of mycelial fungi and actinomycetes-like organisms seemed to tolerate high methanol concentrations (no attempts were made to identify these microbes). A closer examination showed that all failed to grow on methanol in the mineral medium. With methanol concentrations below 7% no real differences were found, although bacteria seem to prefer lower concentrations.

Stationary enrichment cultures were compared with shaken cultures. A soil perfusion apparatus was also employed according to the description by Cripps and Norris (2). (The soil sample is fixed between cotton plugs in a circular glass tube while the medium is forced to pass through and back.) Stationary incubation either turned out to be superior or on difference could be observed at all.

Isolated strains

The pink pigmented Pseudomonas species, also present in the mixed culture, seemed to be very common in nature. It occurred in all of the enrichment cultures and was very easy to detect due to its outstanding pigmentation.

Hyphomicrobia were also relatively common and easy to isolate but grow comparatively slowly and often need growth factors.

However, from the enrichments of sewage sludge, very few different types of colonies developed on the methanol agar plates. These were dominated, not by the pink organism as in other samples, but of a smooth white-yellow colony type. The organism was isolated in pure culture and fulfilled the requirements of growing in the liquid mineral methanol medium without any growth factors. From preliminary growth studies in fermenter cultivations this culture was chosen among the

isolates for the subsequent work on biomass production from methanol. Similar organisms were then found in other samples and could also be recovered from the same source at later occasions.

Apart from these three main types of organisms, a number of other Gram-negative bacterial strains were isolated but not identified.

DISCUSSION

The interactions in the mixed culture TM 20 are certainly more complex than what is possible to understand from the results reported here. None of the pure strains were able to reach the same growth rate as the mixed culture. This is obviously a very common characteristic of mixed cultures. The species in a stable mixed culture are selected during prolonged cultivation in the laboratory (or in nature) and are thus adapted to the conditions provided within the culture. If such a culture is separated into its components the necessary prerequisites for the propagation of individual strains are lost, or the growth rate is reduced due to lack of growth stimulating factors. In a mixed culture of thermophilic methanol utilizing bacteria isolated by Snedecor and Cooney (7) none of the strains were able to grow in pure culture. In the five component mixed culture by Wren et al. (9) only one of the species utilized methanol when it was cultivated as a pure strain, but the growth rate was considerably lower than for the mixed culture. The mixed methane culture by Wilkinson et al. (8) was also strictly dependent on the specific relations within the culture. One of the species, a Hyphomicrobium sp. assimilated methanol which was produced in the culture and otherwise inhibited completely the methane bacteria. The reasons for the behaviour of culture TM 20 in continuous culture were not pursued, but it is possible that essential growth factors or growth stimulating factors do not accumulate to the required concentrations which is the case in batch cultures.

That the individual strains in the mixed culture were unable to grow sufficiently when extracted in a pure form, does not mean that all pure methanol utilizing strains have lower growth rates than in a mixed culture. Several of the pure strains isolated from nature in this investigation, had indeed higher growth rates than the mixed culture. The criteria, used for selecting of the isolates for use in the coming work on biomass production, were growth rate and biomass yield in batch culture. These are the only characters, which in practice can be tested on a strain collection without too many time-consuming experiments and are indeed of great importance. One has though to observe that accumulated knowledge of the process may arise other criteria for strain selection. This means that a continuous work on strain isolation and strain improvement always must be integrated as a natural part of the process development.

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III. DESCRIPTION OF A NEW METHANOL UTILIZING BACTERIUM

SUMMARY

A new methanol utilizing bacterium had been isolated. An electron microscopic investigation does not reveal any unusual structures. The organism is obligately dependent on the one-carbon compounds methanol or methylamine for growth. It is preliminary classified as Methylomonas methanolica. The organism has a high protein content and a well balanced amino acid composition. Crude protein in the biomass is 81.9%, amino acid protein 63%, and essential amino acids 27.2%.

INTRODUCTION

Microorganisms capable of non-autotrophic growth on one-carbon compounds, or on compounds with more than one carbon atoms but containing no carbon-carbon bonds are classified as methylotrophs (2). These are further divided into obligate methylotrophs and facultative methylotrophs.

The obligate methylotrophs are Gram-negative aerobic bacteria. To this group belong all the methane utilizing bacteria described by Whittenbury et al. (31), some of which also are capable of utilizing methanol. The methane utilizing bacteria are characterized by intracytoplasmic membrane systems of two morphologically different types (5,23). Bacteria with type I membrane systems incorporate C_1 -units into cell constituents via the ribulose monophosphate cycle for formaldehyde fixation (14). Bacteria with type II systems use the serine pathway for formaldehyde assimilation (14). Membranes of type I are considered to play a key role in the oxidation of methane, when in bacteria with type II systems the membranes are believed to be required only for electron transport and oxidative phosphorylation (30). Recently have also other Gram-negative obligate methylotrophs been reported, such as bacterium 4B6 (2,3),

bacterium C2A1 (3), organism W1 (4), and Methylomonas M-15 (25). These organisms are, however, not capable of utilizing methane but grow on methanol, methylamine, or dimethylamine. They do not possess any cytoplasmic membrane systems. C₁-units are incorporated into cell constituents via the ribulose monophosphate pathway.

Facultative methylotrophs can in addition to one-carbon compounds also utilize other substrates for growth. Most of the facultative methylotrophs cannot utilize methane for growth, nor do they have the cytoplasmic membrane systems. Methane utilizing bacteria, with the characteristic internal membrane system, have, however, been reported to be capable of facultative methylotrophy (21). Most of the facultative methylotrophs use the serine pathway for incorporation of C₁-units, but the ribulose phosphate pathway has also been discovered in this group (19,27). Facultative methylotrophs are a much more diverse group of microorganisms than obligate methylotrophs. All pink pigmented pseudomonads, so common in nature, belong to this group (1,2,3,11,17,22,28). Hyphomicrobium sp. (10), Gram-positive spore-forming bacteria (19), non-pigmented pseudomonads (8,13,27), non-pigmented Gram-negative non-motile bacteria (2,3), actinomycetes (12), and yeasts (15,20, 24) are also capable of facultative methylotrophy.

The organism isolated previously (section II) is capable of growing in a mineral salts medium with methanol as the sole source of carbon and energy. In this section are its morphological properties and nutritional characteristics investigated. This characterization is not complete from a metabolic point of view, but the data presented give the organism an identity and it is possible to compare it to other methylotrophs. The protein content and amino acid composition was also determined. The high protein content and relatively well balanced amino acid composition predict this strain as a suitable source of SCP.

MATERIALS AND METHODS

Culture

Methylobionas methanolica (see discussion for classification)

Media

MM2 growth medium and MM2 agar medium

Cultivation conditions

Shaking flasks 25-100 ml cultivation volume

Analyses

Optical density estimations.

Nitrogen in biomass.

Amino acids in biomass.

Electron microscopy

After growth, the bacteria were harvested and resuspended in 4% glutaraldehyde in phosphate buffer and centrifuged at 3200 x g for one hour. The bacterial pellet was left over night at +4°C in the fixative and could then be handled as a unit. Before dehydration in increasing concentrations of ethanol the sample was rinsed in phosphate buffer (pH 7.0). In those cases where post-osmication was applied, glutaraldehyde treatment was 2 hours (including centrifugation). The specimen was then rinsed in phosphate buffer, resuspended in Palades 2% osmium tetroxide and centrifuged for one hour. The stable agglomerate of cells thus obtained was rinsed several times in phosphate buffer before dehydration. Epon embedding technique according to Luft (16) was applied. Thin sections were cut with an LKB ultratome, using glass knives, and post stained with uranyl acetate and lead citrate. The samples were examined in a Philips EM 301 G electron microscope, operated at 60 kV.

Growth on different nitrogen sources

MM2 growth medium was used, but, on an equimolar basis, NH_4Cl was replaced by the substance going to be tested.

Growth on carbon sources, other than methanol

The carbon source was added to MM2 salts solution in 0.1% (w/v) or lower (0.05-0.01% for aldehydes). pH was adjusted to about 6.5 with NaOH or HCl when necessary. Absence of growth after 3 weeks of incubation was taken as a criterium of inability to utilize the substrate in question.

Oxidation experiments

Oxidation of different substrates was measured qualitatively by use of an oxygen electrode (see general methods) in a closed glass vessel with magnetic stirring. 9.5 ml MM2 salts solution, saturated with air, was added to the reaction vessel. 0.5 ml of a concentrated washed bacterial suspension in the same salts solution was then added to the system. An immediate rapid decrease in dissolved oxygen was always detected on the recorder, which continuously registrated the course. After approximately one minute the slope of the decreasing dissolved oxygen signal became less steep and had, after another minute stabilized at a very slow reaction rate which represent the endogenous respiration. At that time 0.1 ml of the substrate to be tested was added to a final concentration of 0.1% (% v/v for alcohols and % w/v for other substrates). If the slope of the dissolved oxygen signal then increased and the reaction continued until dissolved oxygen approached zero % of the saturation value, this was taken as a criterium for oxidation of the substrate in question. Methanol was used for comparison.

RESULTS

General description

The new isolate is a strictly aerobic Gram-negative bacterium. The cells are rodshaped, $0.6 \times 1.6 \mu\text{m}$, occurring single or in pairs. A proportion of the cells is motile by means of one polar flagellum, revealed by conventional staining techniques and by electron micrographs. The motility seemed to have decreased after the strain had been kept in the laboratory for some time. Staining with india ink reveals no apparent capsule. The organism is catalase and oxidase positive. Colonies on methanol agar plates are transparent, white to pale-yellow, circular with entire edges, smooth and rather flat. After one day they are punctiform and after six days incubation at 30°C measuring 3-4 mm in diameter.

Electron microscopy

To obtain further information on the morphological characters of the organism, a microscopic investigation was undertaken. Different modes of fixation and staining have been performed. In preparations stained with uranyl acetate and lead citrate it is possible to distinguish cell wall materials as protruding parts from the cell wall (Fig.1 and Fig.2). As shown by others (26,32), structures like these are related to production of capsular or slimy materials. The organism has no visible capsule but it produces an extracellular slime and it is thus very likely that the visible structures are the morphological background to the slime production (see further sections VI and VII). Neither in these preparations (Fig. 1 and Fig.2), nor in preparations stained only with uranyl acetate (Fig.3), any morphological characters unusual to bacteria in general, were found. The dark inclusions (Fig.3) represent polyphosphate accumulations and some of the light ones might show lipid storage material (18). The granular cytoplasm is typical for bacteria.

Growth

The optimum growth temperature is 30°C. At 40°C the culture grows in the first transference but fails to grow in subcultures. pH optimum is 6.3-6.9 but growth occurs between pH 5.3 and 8.5. For maximum growth rate the methanol concentration should be kept under 0.5% (v/v). At concentrations above 1%, growth rate and yield are drastically reduced and no growth occurs at 6% methanol. In shaken cultures growth is dispers, with no visible pigment production, but centrifuge pellets of the organism have a faint brown-pink appearance. Doubling time in shaken cultures (MM2 growth medium, 30°C, 0.3% methanol, no pH control) is 1.5-1.8 h. No growth factors are required.

Nitrogen sources for growth

Ammonium chloride was routinely used in the ordinary medium but the organism utilizes nitrate, casamino acids, yeast extract, and urea almost equally well. Nitrite also support growth but is inhibitory over 0.1 g·l⁻¹. When the strain is cultivated on nitrate or ammonium salts detectable amounts of nitrite is formed in the medium, which also is reported for other methylotrophs (31).

Carbon sources for growth

Methanol and methylamine hydrochloride were the only carbon sources that could be utilized for growth. When the cells were transferred from methanol to methylamine, a lag phase of 10-12 days was observed before growth started. After a few subcultures on methylamine, the lag phase was reduced to 1 day but growth was still very slow (with a doubling time of 20 h). In transfers from methylamine to methanol, the growth rate approached normal values after a couple of hours. Growth on methylamine thus required an adaptation period, indicating the presence of an inducible enzyme, whereas the ability to utilize methanol is constitutive. The organism is also able to utilize methylamine as carbon

and nitrogen sources simultaneously, since it grows in the mineral medium supplemented with methylamine but without ammonium chloride.

Other substrates tested for growth were: methane, n-alcohols (ethanol, propanol, butanol, and petanol), aldehydes (formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, and valeraldehyde), carboxylic acids (formic, acetic, propionic, butyric, and valeric acids), organic acids (citric, lactic, pyruvic, succinic, fumaric, α -ketoglutaric, and oxalic acids), sugars and related compounds (glucose, fructose, lactose, sucrose, maltose, mannose, xylose, arabinose, glycerol, sorbitol, mannitol, and inositol), complex media (yeast extract, nutrient broth, and peptone), and casamino acids.

Oxidation by washed cell suspensions

Washed cell suspensions of the organism oxidize ethanol, propanol, butanol, pentanol, formaldehyde, acetaldehyde, propionaldehyde, butyraldehyde, valeraldehyde, and formic acid. Other carboxylic acids (acetic, propionic, butyric, and valeric acids), organic acids (citric, lactic, succinic, fumaric, α -ketoglutaric, and oxalic acids) or sugars (glucose) were not oxidized. Methanol grown cells did not oxidize methylamine.

Protein content

The amino acid composition and protein content of cells were analysed. The figures summarized in Table 1 and Table 2 are maximum values.

Table 1. Amino acid composition of the methanol utilizing organism

Amino acid	g/100 g biomass	Amino acid	g/100 g biomass
Lysine	4.830	Arginine	3.721
Threonine	3.388	Cystine	0.057 ^a
Methionine	1.475 ^a	Tyrosine	2.429
Tryptophan	- ^a	Alanine	5.261
Isoleucine	3.373	Aspartic acid	7.020
Leucine	5.639	Glutamic acid	7.770
Valine	4.282	Glycine	4.295
Phenylalanine	2.915	Proline	2.415
Histidine	1.515	Serine	2.637

a) Methionine and cystine are partly destroyed by the acid hydrolysis and tryptophan is completely destroyed.

Table 2. Protein content of the methanol utilizing organism.

	g/100 g biomass
Total nitrogen (N_T)	13.1
Crude protein ($N_T \cdot 6.25$)	81.9
Amino acids according to analysis	63.0
Estimated losses during hydrolysis (tryptophan, cystine, methionine)	1.3
Total amino acids (N_{AT})	64.3
Essential amino acids	27.2
Non-protein nitrogen ($N_T \cdot 6.25 - N_{AT}$)	17.6

DISCUSSION

The organism described here is obligately dependent on methanol or methylamine for carbon and energy sources. This organism does not grow on methane, nor has it the characteristic internal membrane system of the methane utilizers. Using only the one-carbon compounds methanol and methylamine classifies this organism as an obligate methylotroph and, in this respect, it is similar to strain W1 described by Dahl et al. (4). Washed cell suspensions of strain W1 have a similar oxidation pattern as this organism except for the inability of strain W1 to oxidize the primary aldehydes above formaldehyde. The presence of an inducible methylamine dehydrogenase was proved in organism W1. The growth and respiration studies reported here, indicate that this organism also has an inducible enzyme for the oxidation of methylamine. Tsugi and Dostálková have shown that crude extracts of the organism described here contain a methanol dehydrogenase, a formaldehyde dehydrogenase, and a formate dehydrogenase (29). The methanol dehydrogenase had a broad substrate specificity towards primary alcohols and oxidized also formaldehyde. The formaldehyde dehydrogenase had likewise a broad specificity to primary aldehydes. These results verify the oxidation studies in this investigation. It is not yet known whether this organism use the serine pathway or the ribulose monophosphate pathway for incorporation of C_1 units into cell constituents.

On basis of its obligate dependence of the one-carbon compounds methanol and methylamine this organism is preliminary classified as Methylomonas methanolica. Grouping of obligate methylotrophs under the common prefix "Methylo-" was first proposed by Foster and Davis (9) and then by Whittenbury et al. (31). These authors considered, however, only bacteria capable of utilizing methane and methanol. One might argue that the ability to use methylamine exclude such organisms from the ranks of "Methylo-", a standpoint adopted in the description of strain W1. A definitive classification can certainly not be made until more is known about the biochemical properties of this organism and

of similar isolates. The name Methylomonas methanolica will be kept for the present so no confusion will arise about the organism which is considered. The strain has been designed M. methanolica in several publications and reports (6,7,29).

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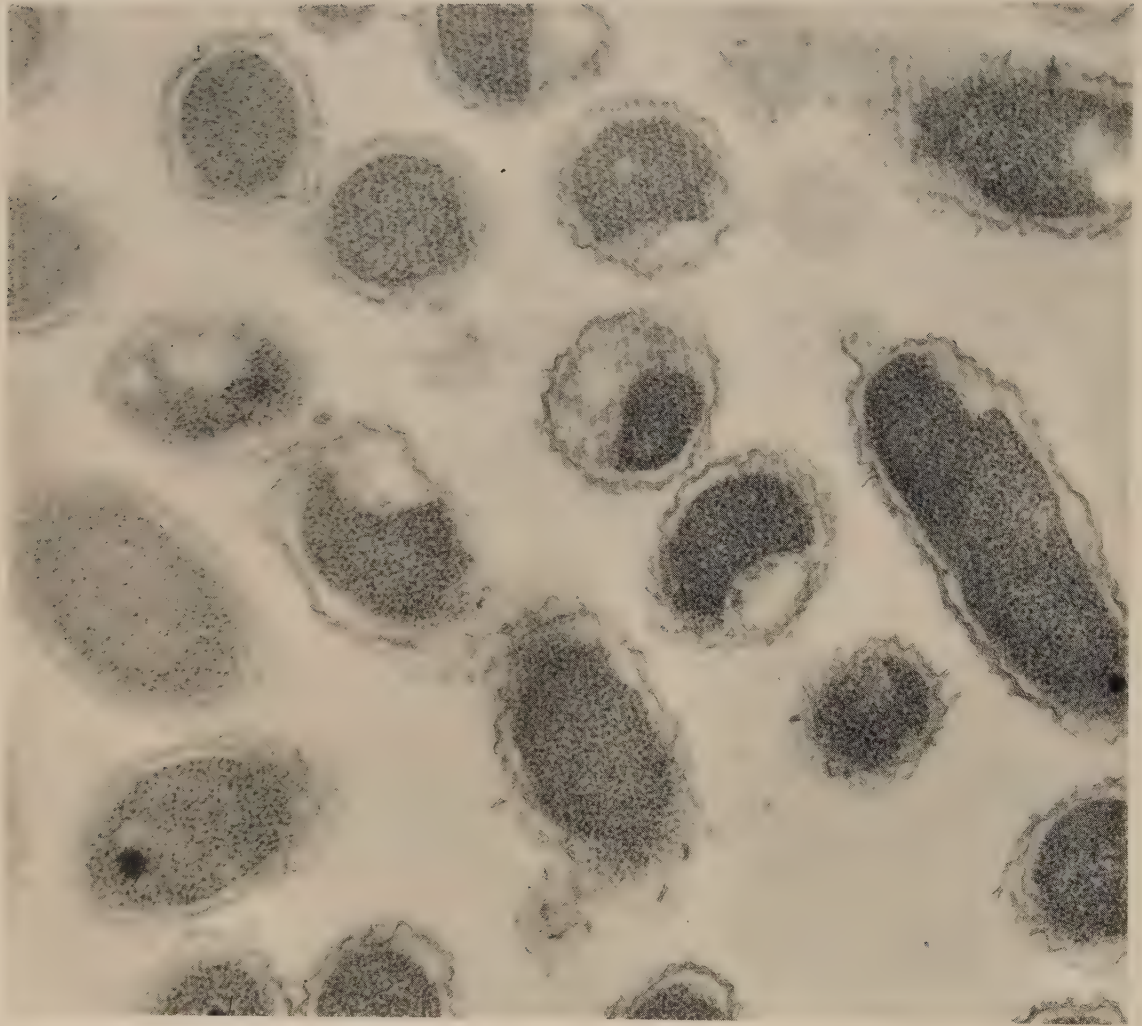


FIG 1. A GLUTARALDEHYDE-FIXED PREPARATION OF THE METHANOL UTILIZING BACTERIUM, STAINED WITH URANYL ACETATE AND LEAD CITRATE. x50 000.

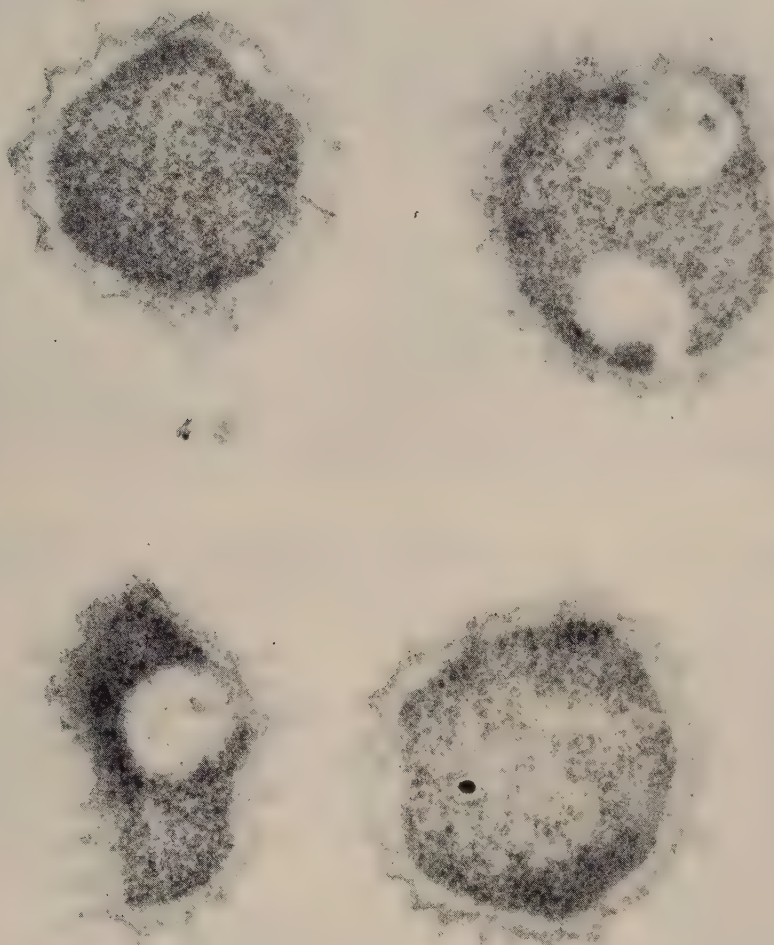


FIG 2. A GLUTARALDEHYDE-FIXED AND POST OSMICATED PREPARATION OF THE METHANOL UTILIZING BACTERIUM, STAINED WITH URANYL ACETATE AND LEAD CITRATE. x 91 000.

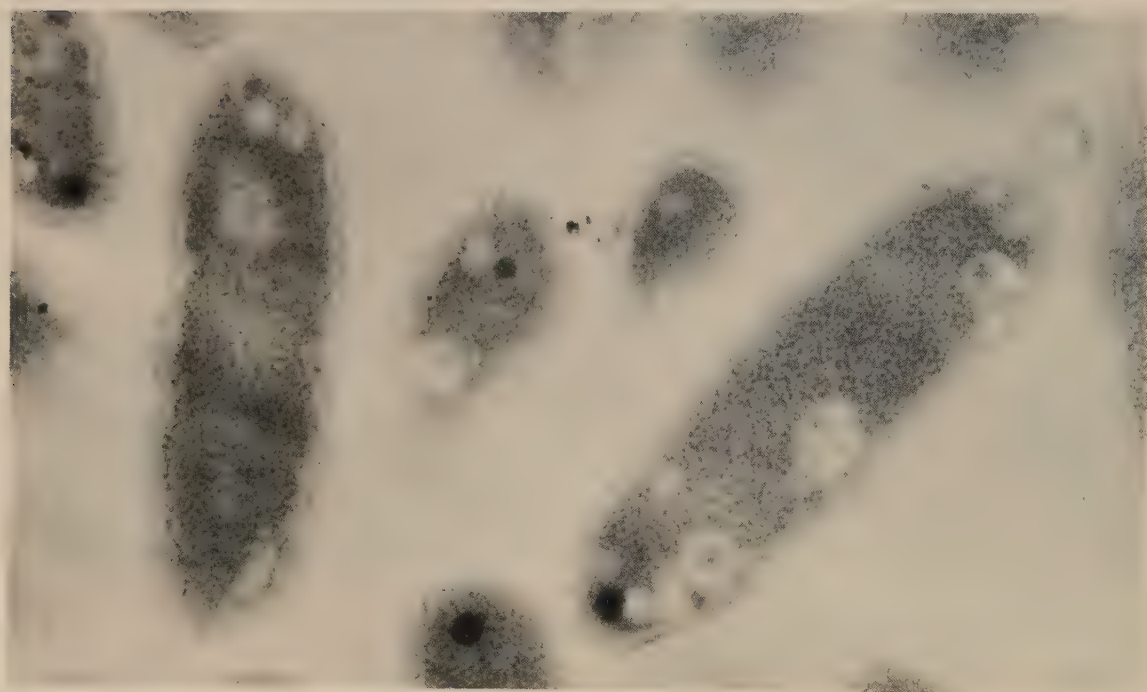
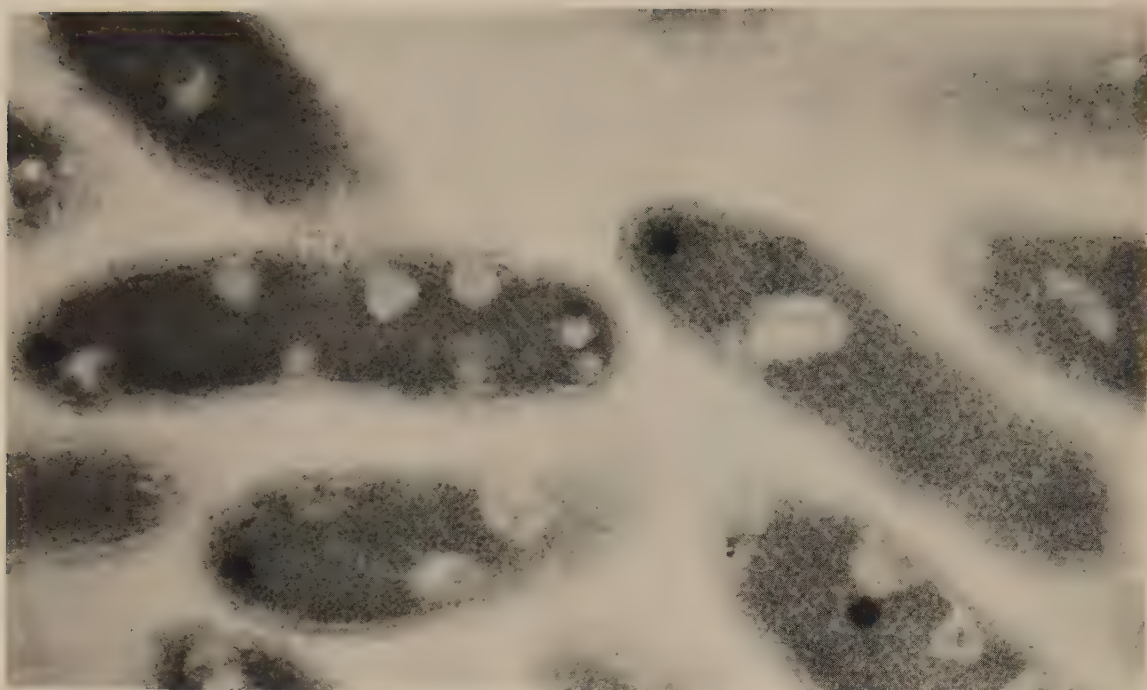


FIG 3. A GLUTARALDEHYDE-FIXED PREPARATION OF THE METHANOL UTILIZING BACTERIUM, STAINED WITH URANYL ACETATE, x 54 600.

IV. OPTIMIZATION OF BIOMASS PRODUCTION FROM METHANOL

SUMMARY

A specific mineral medium was devised for maximal biomass production from Methylomonas methanolica. The importance of the relative and absolute concentrations of minerals was demonstrated. In batch culture the maximum productivity increased from $1.55 \text{ g} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ to $2.63 \text{ g} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ and the final biomass yield from $7.2 \text{ g} \cdot \text{l}^{-1}$ to $18.0 \text{ g} \cdot \text{l}^{-1}$ when the new medium was used. Comparative studies on biomass production in batch culture, chemostat continuous culture, semi-continuous culture, and turbidostat culture showed that a three times higher productivity was achieved in all types of continuous culture than in the complete batch process.

INTRODUCTION

Optimization of microbial product formation is of the utmost importance for the development of new fermentation processes. A lack of reliable and continuous analytical methods and an incomplete knowledge of the interrelation between the various growth parameters have resulted in a much slower development to date in the process control of fermentations compared to the development of chemical processes.

In this section, the discussion is restricted to biomass production from Methylomonas methanolica in respect to medium composition and cultivation method. The effect on growth and productivity of the medium composition and especially the effect of the relative concentrations of minerals is investigated. The ultimate goal was to devise cultivation conditions of such a sort that all components in the medium are present in optimal relations and so that these relations are maintained by dosing of a salts solution during the entire cultivation period.

Different types of cultivation procedures is also studied. Batch culture and individual types of continuous culture are compared on basis of biomass production and substrate utilization.

MATERIALS AND METHODS

Culture

Methylomonas methanolica

Media

MM2 growth medium

W8 + W9 are the growth medium, respectively the dosing medium, developed in this investigation. The composition of these are given in general methods.

Cultivation conditions

Biotec (2-4 l) fermentation unit equipped for batch culture or for continuous culture. Aeration conditions correspond to an OTR of $300 \text{ m mol O}_2 \cdot \text{l}^{-1} \cdot \text{h}^{-1}$.

Analyses

Bacterial dry weight.

Methanol in growth medium and in flow medium for continuous culture.

Metals and phosphorus in media and biomass.

Nitrogen in biomass.

Ammonium nitrogen in media.

RESULTS

Substrate optimization

In order to optimize growth in batch culture a growth medium was first made. The composition of this medium corresponds to the composition of the bacterial cell in such a way that the relative proportions between individual elements are the same.

As a basis for making up the recipe, analyses of cells, harvested in the logarithmic phase, was used. This medium was then used to evaluate the optimal total concentration of the medium, using maximal growth rate and the length of the lag phase as criteria. The conditions for an optimal growth medium are fulfilled when the concentration of the elements corresponds to 20% of the total consumption in the batch process. In media containing a three times higher concentration, significant prolongation of the lag phase and depression of the growth rate were observed.

The next step was to maintain a constant composition of the medium during growth. On the basis of biomass and media analyses, it became clear that a number of the most important elements are consumed in a constant relation to the consumption of nitrogen. For that reason, ammonium salts were used as the nitrogen source, which made it possible to compensate for the consumed nitrogen by dosing an ammonium hydroxide solution on the basis of the pH changes. A solution, containing all other elements in the appropriate relation to nitrogen, was dosed in parallel to the dosing of ammonium hydroxide. In Table 1, the composition of the growth and dosing media is shown. This procedure enabled maintenance of constant concentrations and relations between N, P, Mg, Ca, Fe, Zn, Cu, and Mn during the logarithmic growth phase with a relative error lower than $\pm 5\%$. In the late declining phase, however, differences in the relation between nitrogen and the other elements were observed.

Table 1. Composition of growth medium and dosing medium for cultivation of Methylomonas methanolica at balanced conditions.

Elements	Ratio $\frac{\text{Element}}{\text{N}} \cdot 100$
N	100.0
P	25.0
S	9.0
K	2.0
Mg	2.0
Ca	1.5
Fe	2.5
Cu	0.08
Zn	0.08
Mn	0.01
Mo	0.001
Co	0.001

Relative concentrations are given for individual elements in relation to nitrogen ($N = 100$). The total concentration of nitrogen in the growth medium was $0.4 \text{ g} \cdot \text{l}^{-1}$ and for the dosing medium, $20 \text{ g} \cdot \text{l}^{-1}$.

Fig. 1 presents a comparison of growth parameters at balanced and unbalanced cultivation conditions, respectively. The MM2 growth medium was used in a conventional batch process as an example of unbalanced cultivation conditions. In this case, the exponential phase was shorter and the declining phase started earlier. Maximum yield achieved was $7.2 \text{ g} \cdot \text{l}^{-1}$.

Balanced cultivation conditions, on the other hand, gave a longer logarithmic phase and the final yield reached $18.0 \text{ g} \cdot \text{l}^{-1}$. The mean productivity increased from 0.30 to $0.75 \text{ g} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ and the maximum productivity increased from 1.55 to $2.63 \text{ g} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$, which was the highest productivity obtained for the aeration condition used. The yield coefficient for methanol $\frac{Y_x}{s}$ (g biomass produced/g methanol consumed) was also affected by the new medium. $\frac{Y_x}{s}$ max (measured during logarithmic growth) increased from 0.34 to $0.46 \text{ g} \cdot \text{g}^{-1}$.

Continuous cultivation techniques

The growth parameters in balanced batch cultivations, as presented in Fig. 1, were used as a basis for these studies. Special attention was paid to areas of dilution rate which corresponds to high productivity of biomass.

A chemostat type of continuous culture was carried out, using either methanol or oxygen as limiting factors (Fig.2). In both cases the maximum productivity achieved was $2.3 \text{ g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$ at a dilution rate of 0.24 h^{-1} and at a biomass concentration of $9.6 \text{ g}\cdot\text{l}^{-1}$. The yield coefficient for methanol was $0.34 \text{ g}\cdot\text{g}^{-1}$ at conditions giving maximum productivity.

Semi-continuous culture was studied using a low number of withdrawals ($n = 1, 2, 3$). With two withdrawals, maximum productivity was $2.4 \text{ g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$ at a mean dilution rate of 0.24 h^{-1} and at a biomass concentration of $10.0 \text{ g}\cdot\text{l}^{-1}$. The mean specific growth rate (μ) was 0.27 h^{-1} and the yield coefficient for methanol was $0.35 \text{ g}\cdot\text{g}^{-1}$ (Fig.2).

A turbidostat type of continuous culture was accomplished by three different principles of flow control. In the first case, the flow control device was a turbidimeter constructed at the laboratory. A measurement cell for light absorption was placed in a bypass medium stream and connected by fibre optics to a photometer and a light source. In the other two cases the flow was controlled proportionally to either methanol or nitrogen consumption. In all three types of flow control, a maximum productivity of $2.5 \text{ g}\cdot\text{l}^{-1}\cdot\text{h}^{-1}$ at a biomass concentration of $9.26 \text{ g}\cdot\text{l}^{-1}$ and at a dilution rate of 0.27 h^{-1} was achieved. The yield coefficient for methanol was $0.36 \text{ g}\cdot\text{g}^{-1}$ (Fig.2).

DISCUSSION

According to Nicholas (4) the following elements are essential for microorganisms: N, P, Mg, Ca, S, Fe, Cu, Zn, Mn, Mo, B, Cl, K, Na, Co and V. For optimal growth, N, P, Cl, Mg, Ca, and S must be available in the concentration range 10^{-2} - 10^{-4} M. The need for other elements is in the range 10^{-6} - 10^{-8} M. The rate of uptake of nutrients depends on the ions in which the elements are present and also on the chemical and physical environment in which the cells are growing. The properties of the substrate are also influenced by pH because of the effect on solubility and dissociation.

In higher plants has a constant relation between the relative concentration of minerals, available in the growth medium and the concentration of minerals in the plant tissue been established (2,3). Data from the literature indicate that the proportion of minerals in relation to nitrogen is, with few exceptions, relatively constant in bacteria. The composition of the minerals in growth media, used by different investigators, shows on the other hand surprisingly wide variations and the data clearly show that criteria other than cell composition have been used, when making up the recipe for the relative concentrations of minerals.

Table 2, which summarizes values from the literature, shows the relative composition of bacterial cells and the corresponding compositions of media.

Table 2. Relative concentrations of individual elements in bacterial cells and commonly used cultivation media.

Elements	Cells		Media	
	X	n	X	n
N	100		100	
P	23	25	176	66
K	14	24	201	65
S	8.9	7	59	70
Mg	4.9	27	15	74
Na	3.2	8	66	41
Ca	3.0	25	11	47
Cl	2.5	3	123	29
Fe	0.3	20	2.2	60
Zn	0.14	6	0.13	26
Cu	0.03	21	0.04	27
Mn	0.05	20	0.15	26
Co	0.003	2	0.02	13
Mo	0.002	3	0.09	17
B	0.006	11	0.01	9

The figures are mean values from published data for Gram-positive and Gram-negative bacteria grown in mineral media. $\bar{X}$ is the proportion of the element in relation to nitrogen, n is number of references.

A general model for making up a medium for bacterial growth does not apparently exist. Nor has a dosing medium, intended to maintain the absolute and relative concentrations of minerals during cultivation, been described. The medium used for batch culture is usually used also as a flow medium for continuous culture, which sooner or later could lead to the exhaustion of one or several growth factors, if the concentration does not correspond to the rate of uptake of the various elements.

The results reported here show the importance of a balanced mineral medium, the composition of which is based on the specific cell composition. The dosing medium not only enabled a maintaining of a constant environment during growth, but also promoted a longer growth phase. If the required amounts of nutrients had been present in the growth medium from the beginning toxic effects would have depressed both growth rate and final yield.

In evaluation of the different types of cultivation procedures the new medium was used. All types of continuous culture gave better results than the batch culture. A comparison of the maximum and mean productivity values in batch culture shows clearly that only a fraction of the total production potential is utilized. The productivity in continuous culture was more than three times higher than the mean productivity in batch culture. The substrate utilization was also more effective in continuous culture than in the complete batch culture. Dostálek and Molin have shown (1) that the yield coefficient for methanol varies with the growth rate of Methylomonas methanolica, which accounts for the higher maximal value in batch culture, where the growth rate is higher than was the case in continuous culture.

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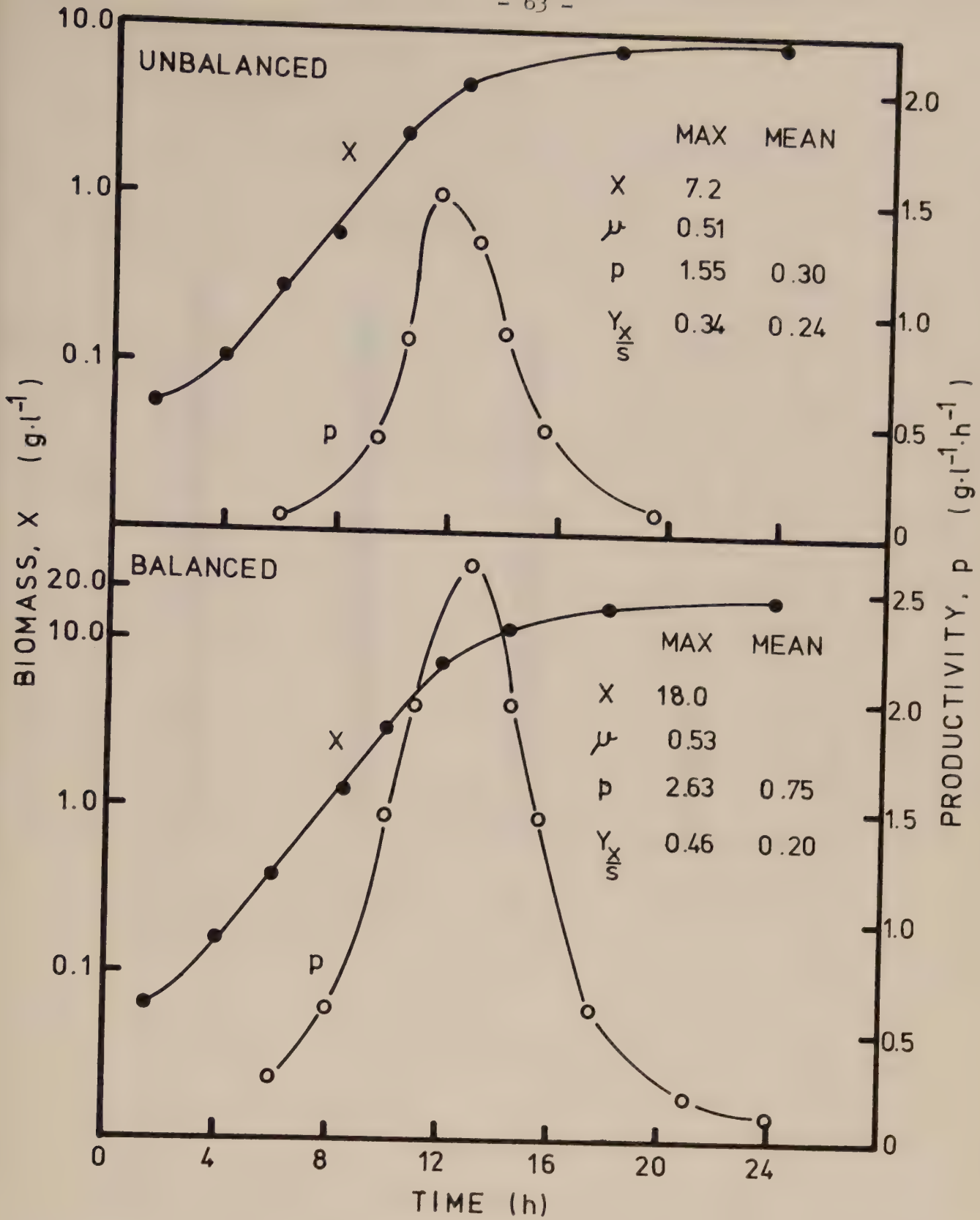


FIG 1. GROWTH AND PRODUCTIVITY OF METHYLOMONAS METHANOLICA IN BATCH CULTURE AT BALANCED AND UNBALANCED CONDITION.

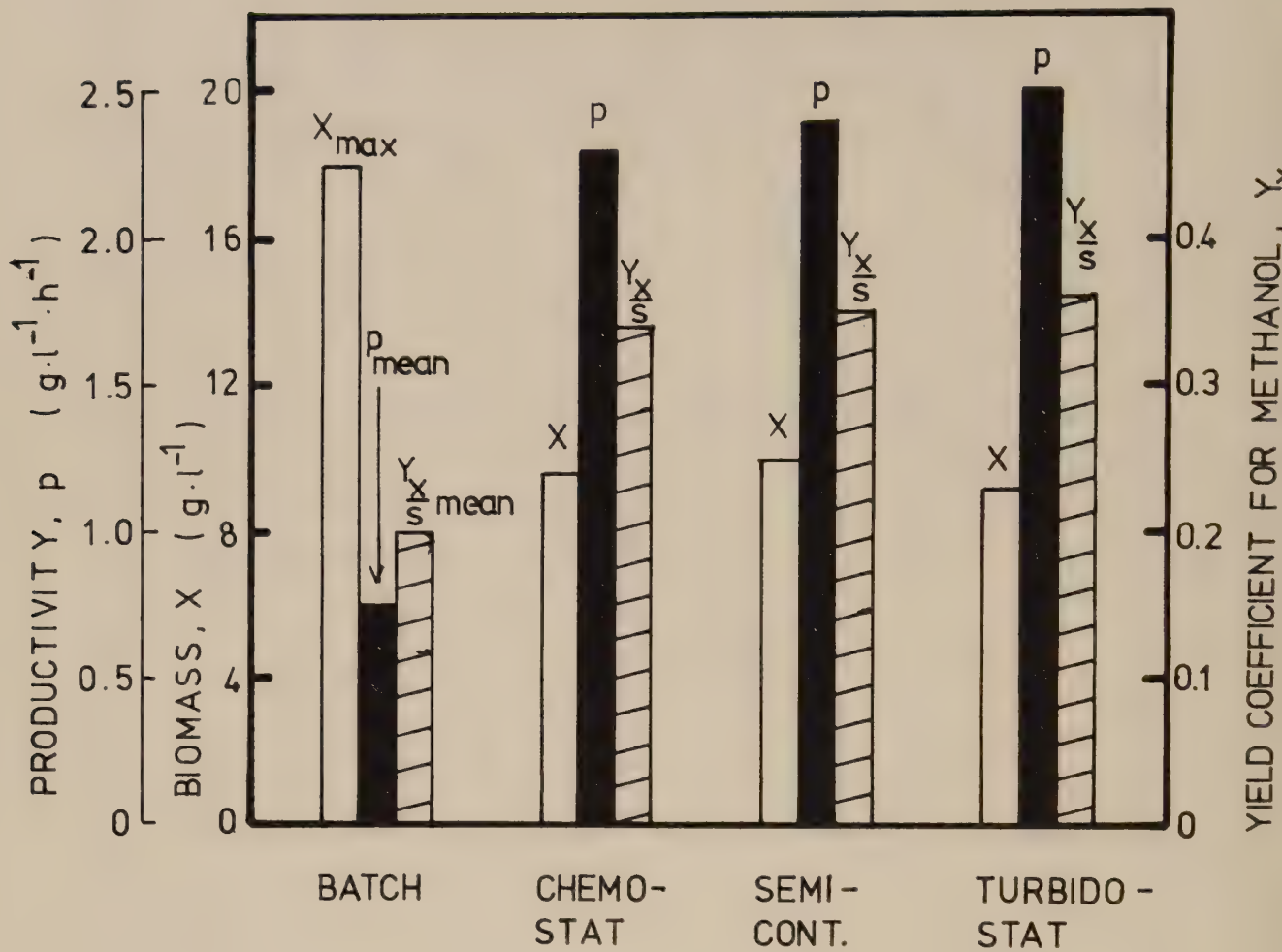


FIG 2. COMPARISON OF BIOMASS CONCENTRATION, PRODUCTIVITY, AND YIELD COEFFICIENT FOR METHANOL IN BATCH CULTURE AND DIFFERENT TYPES OF CONTINUOUS CULTURES OF *METHYLOMONAS METHANOLICA*.

V. METHODS FOR OBTAINING MUTANTS WITH HIGHER TEMPERATURE TOLERANCE FROM METHYLOMONAS METHANOLICA

SUMMARY

Methylomonas methanolica can be adapted to growth at 37°C retaining the same growth rate as the wild strain has at the temperature optimum of 30°C. This character is lost if the adapted culture is transferred to 30°C again. By means of mutagenic treatment a stable variant of M. methanolica was isolated with temperature optimum at 35-37°C and maximum growth temperature at 43°C compared with 40°C for the wild type. The results imply that further extension of the temperature tolerance for M. methanolica is impossible.

INTRODUCTION

As already discussed in the general introduction it is of great importance in all SCP processes to use a thermotolerant strain. The purpose of the work presented in this section was to investigate different methods for obtaining thermotolerant variants of Methylomonas methanolica, if possible with retaining of the same growth rate as for the wild strain.

The formation of temperature tolerant mutants seems to be of a highly strain specific character being a consistent phenomenon or not occurring at all. A number of mesophilic Bacillus species (B. subtilis; B. cereus; B. megaterium; B. circulans) investigated by Allen (2) were able to produce mutants capable of growth at 55-60°C, when other species (B. macerans; B. coagulans; Pseudomonas sp.; Escherichia sp.; Aerobacter sp.; Lactobacillus sp.; Streptococcus sp.; Mycobacterium sp.; and actinomycetes) did not form any such mutants at all. Pseudomonas fluorescens (7) is also reported to produce mutants with a maximum growth temperature at 54°C compared with 37°C for the wild type, while, in the same investigation Escherichia coli

and Pseudomonas aeruginosa were unable to create any temperature tolerant mutants. Mesophilic mutants from the psychrophiles Micrococcus cryophilus (13,15) and Bacillus psychrophilus (10) have also been obtained. Successful experiments to adapt bacteria to higher temperatures have as well been performed. Bacillus subtilis, normally grown at 37°C and with a maximum growth temperature of 53-55°C, could be adapted to grow at 72°C by slowly increasing of the temperature step by step (8).

Neither the biochemical nor the genetical background to thermophili are clear. A review of the literature reveals many definitive differences between mesophiles and thermophiles. Though there are extensive evidences on higher heat resistance in thermophilic ribonucleic acids, ribosomes (9), and enzymes (14) the molecular basis for this character has not yet been resolved. In fact, these compounds are surprisingly similar to their mesophilic equivalences. Mechanisms, like a stabilizing action of cross-linking cations (12) and of the cell membrane (4,6), may also contribute to thermal resistance. Another explanation for thermophili is an increased capacity for rapid resynthesis of heat-denatured compounds (2,5). It is also proposed that facultative thermophilic organisms have two types of metabolism functions at different temperatures, as transference from low to high temperature or vice versa requiring incubation at an intermediate temperature with protein synthesis during this time (3).

Thermophili is undoubtedly a complex of many mechanisms which certainly can not be explained in generalized terms. In fact, microorganisms are growing over a total temperature range of nearly 100°C. It is easy to visualize that the growth limiting temperature is determined by the most heat-labile component in the cell, whether this is a single essential enzyme, not being active over a certain temperature, or it is the protein synthesis not being capable of keeping up with a faster turn over rate.

In order to investigate if M. methanolica was susceptible to changes in its temperature tolerance range, the strain was subjected to adaptation to higher temperatures, in searching for spontaneous mutants, and induction of mutants with a chemical mutagenic agent.

MATERIALS AND METHODS

Culture

Methylomonas methanolica

Media

MM2 growth medium and MM2 agar medium.

Cultivation conditions

Shaking flasks, 25-100 ml cultivation volume.

Analyses

Optical density estimations (Linson 3, Turner).

Temperature gradient incubator

In order to be able to carry out the search for strains with higher temperature tolerance, it was necessary to have a quick and simple method for routine determinations of growth as a function of temperature.

In a block of aluminium measuring 60x10x10 cm, isolated against the environment with a wooden case containing Frigolite, a linear temperature gradient was obtained by pumping thermostated hot water through internal loops at one end of the block and cold water at the opposite end. Two parallel rows of each 20 holes ($\varnothing$ 1.6 cm; depth 5 cm) designed for test tubes were placed in the direction of the gradient. In this way it was possible to measure bacterial growth in liquid medium at 20 different temperatures in duplicate. The aluminium

block also contained two length going channels (48.5x1.2x1.2 cm) for incubation on solid substrate. The temperature could be checked at five points along the gradient by insertion of thermometers in separate holes. The temperature in the test tubes were checked and found to agree perfectly with the outside check points. A total temperature range of 30°C was usually applied with end points at 20°C and 50°C respectively. Similar devices have also been described by others (11).

For determination of temperature optimum curves a homogeneous bacterial suspension in fresh MM2 growth medium was prepared from a previously shaken culture. Three ml of this suspension were filled into each of 40 test tubes with metal caps. After 40 hours' of incubation in the gradient, optical density of each sample was measured and a temperature optimum curve was constructed. It is though necessary to make the interpretation of a temperature curve obtained in this way with caution. The method does not give any direct information on growth rate. It is thus the shape of the curves rather than the height of the peaks that gives information on the temperature optimum.

The channels designed for incubation on agar medium were sterilized with 70% alcohol and burnt. 50 ml of melted agar were filled into each of the channels, preheated to 45°C, and then allowed to solidify. The inoculum was then spread on the agar surface. A sterilized glass lid covered the channels thus preventing contamination and evaporation from the agar. The lid was heated from above with "heating tape" to avoid condensing of water on the inside. Incubation lasted for six days.

Mutagenic treatment

As mutagen, N-methyl-N'-nitro-N-nitrosoguanidine (MNNG) was used. The general procedure described by Adelberg et al. (1) was followed with some slight alterations.

a) As the mutagenic effect varies with the surviving fraction of cells, a killing curve was prepared as follows: A shaken culture of M. methanolica was harvested in the logarithmic

growth phase, centrifuged and washed twice in MM2 salts solution. The culture was resuspended in 25 ml of MM2 salts solution to an approximate density of $2 \cdot 10^8$ cells $\cdot$ ml $^{-1}$ and pH was adjusted to 6.0. The culture was placed in a shaker at 30°C. At zero time MNNG solution was added to a final concentration of 100 μ g $\cdot$ ml $^{-1}$. Samples were selected at intervals, immediately diluted 100 times in MM2 salts solution and spread on agar plates for viable count. Viable count is normally not made on M. methanolica because of the organism's irregular behaviour on agar plates, probably due to aggregation of cells. A great number of dilutions and parallel plates were, however, made in order to obtain a reliable mean value. The figures obtained may not represent discrete organisms but rather spread units.

During these conditions M. methanolica is killed after exposure to MNNG as illustrated in Fig.1. On the semilogarithmic plot the initial steep slope shows one fraction of sensitive cells followed by an intermediate part and finally there is a more resistant fraction of cells. For the following experiments an exposure time of 4 minutes was chosen in order to obtain a 25% survival.

b) For the mutagenic treatment the culture was prepared in the same manner as in a). After 4 minutes incubation with the mutagen, the suspension was rapidly filtered through a 0.45 μ m Millipore membrane filter and washed three times with 10 ml water. The filter paper was transferred into a shaking flask with 25 ml MM2 growth medium. By shaking, the cells were easily removed from the filter and a homogeneous suspension was obtained. The whole procedure was carried out under sterile conditions.

Viable count was routinely made before and after mutagenic treatment. The proportion of surviving bacteria varied between 20% and 40% in different experiments. Exposure time for MNNG could though vary because the filtration step was difficult to control. However, no correlation between total exposure time

and surviving fraction was found in these experiments. This probably only illustrates the lack of reproducibility in viable count determinations.

After transfer to growth medium the MNNG treated cells were grown over night at the temperature that was optimal before the mutagenic treatment, in order to allow phenotypic expression.

RESULTS

Adaptation

The temperature optimum for M. methanolica is 30-31°C and maximum growth temperature is 40°C as shown in Fig.2. These results were obtained from the temperature gradient incubator and were in good agreement with experiments in shaking flasks (section III). The adaptation procedure was carried out by slowly increasing the temperature from 30°C, usually with 1°C in every new step. As a standard for the degree of adaptation, the growth rate in the logarithmic phase, based on optical density measurements, was used. If the growth rate was lower than normal (growth rate in shaking flasks is normally 1.5-1.8 h at 30°C) additional transferences to fresh media were made at the same temperature until the generation time for the adapted culture reached the same value as for the control. Between every temperature increase the strain was transferred to solid substrate for contamination control. This procedure does not involve any deliberate selection since a fairly big inoculum (10-20%) was used in every transfer.

M. methanolica could thus be adapted to 37°C, retaining the same growth rate as it has at 30°C (Fig.3) but the culture seemed to reach the stationary phase just a little earlier. If the organism, instead of being slowly adapted, is directly transferred from 30°C to 37°C, growth is no longer logarithmic but linear (Fig.3). The adapted culture also loses part of its ability to grow at 37°C after a few cultivations at 30°C, or if it is kept at storing temperatures between 4-8°C.

After a great number of transferences at 38°C, alternating between liquid and solid substrate, the adapted culture has still significantly lower growth rate than the original culture has at 30°C. Attempts to extend the upper growth limit with the adaptation procedure were unsuccessful.

Spontaneous mutants?

The simple method for production of spontaneous mutants of P. fluorescens (7), referred to earlier, has also been tested on M. methanolica. Numbers of shake flasks were inoculated with varying amounts of cells ($10^8 \text{ cells} \cdot \text{ml}^{-1}$ - $2 \cdot 10^9 \text{ cells} \cdot \text{ml}^{-1}$) and incubated at 45°C. No growth could be detected even after prolonged incubation. Transference to solid substrate did not give any colony formation either. One can conclude that if M. methanolica produces spontaneous temperature tolerant mutants at all, they must be present in a very low proportion and not easy to detect in a suspension with a majority of wild type cells, unless a very special selection procedure is applied.

Selection of temperature tolerant mutants after mutagenic treatment

After mutagenic treatment and phenotypic expression as described in materials and methods, the culture was spread on the agar strips in the temperature gradient incubator. The continuous bacterial growth formed a clear front line at the maximum growth temperature. The strips were screened for single colonies above the upper temperature growth limit for the culture.

The reason for incubating the culture in a continuous temperature gradient was the assumption that it would be conceivable to find possible mutants even if their upper temperature limit had been only slightly raised. In those cases, where no individual colonies could be found outside the confluent bacterial growth, the culture was used either unselected in the next mutagenic treatment or the front line of bacteria at maximum temperature was isolated. Between each mutagenic treatment a temperature optimum curve and a growth curve were measured for the isolated culture or for the unselected

culture as described above. If the temperature optimum had changed, the routine incubation temperature was changed correspondingly as well. The fraction that showed the highest temperature optimum was prepared for a new mutagenic treatment. In Fig. 4 a flow diagram presents the way of mutagenic treatment and selection which finally leads to the isolation of a strain with elevated temperature optimum. In practice this scheme had several branches, not described here, leading to similar results or no ones. Fig. 5 shows temperature optimum curves for the different isolates after the first, the fourth and the fifth mutagenic treatment. The last two curves have a broad optimum plateau ranging up to 35°C, indicating a mixture of mutants and wild types. It is also evident that no significant increase of the temperature optimum has occurred during the last mutagenic treatment.

After the last mutagenic treatment and isolation of a single colony the culture was subjected to cultivations at 40°C and 45°C. This treatment seemed to induce drastic changes in one single step. After the first cultivation at 40°C the temperature optimum curve shows a completely different pattern than earlier (Fig. 6). The optimum range is extended to 37°C and the maximum growth limit elevated to 43°C. The maximum optical density had also increased (the OD values in Fig. 6 are not directly comparable to those in Fig. 5 because two different instruments were used, see general methods). Additional cultivations (Fig. 6) at 40°C seemed to amplify these properties. The difference in optical density can partly be explained by higher tolerance against low pH in the isolate, thereby promoting a longer growth phase (see section VI.). Later examinations have also shown that the relationship between dry weight and optical density is lower for the isolate than for the wild type.

Simultaneously with the first change of temperature pattern a new colony type appeared. A great part of the population showed larger and more mucoid colonies. In this case additional cultivations at 40°C also seemed to amplify the new property.

After further shock cultures at 45°C the new colony type was isolated in pure culture. The strain was at the beginning unstable and throw regularly off colonies of varying morphology among those typical wild type of colonies. Temperature optimum curves from this period also showed that the culture was not homogeneous, as reflected by two optimum peaks. Through deliberate selection of the new colony type, a strain (M13V) was finally obtained, which appeared to be completely stable. After two years in the laboratory it still retains its characters, which are temperature optimum at 35-37°C and maximum growth limit at 43°C.

DISCUSSION

1. The ability of M. methanolica to adapt to a good growth at 37°C was clearly established, but it was not possible to achieve a complete adaptation at 38°C, although the adapted strain grew better at this temperature than a non adapted culture. The adaptation procedure did not influence the maximum growth limit, which in fact was the case with Bacillus subtilis (8). Both species, however, lost their temperature tolerance on cultivations at lower temperatures.

2. By using the same procedure which was successful for Pseudomonas fluorescens (7), that means incubation at 45°C, 5°C above the upper growth limit without application of mutagenic agents except for heat itself, the only result was a complete killing of the whole culture.

3. After five mutagenic treatments it was indeed possible to isolate a variant of M. methanolica with extended temperature tolerance. Subjected to 40°C the culture grew perfectly in repeated transferences and it survived and even grew (although poorly) at 45°C which certainly had not been possible earlier. Thus, the mutagenic treatment had induced changes in the culture, as already shown in the temperature optimum curves (Fig.5) but these characters were not fully expressed until selected. In fact, typical mutant colonies were present in the culture

at low frequency already after the fourth mutagenic treatment. It is therefore doubtful if the last treatment has had any additional positive effect.

On the basis of these results one can conclude that it is probably very hard, if possible at all, to extend the temperature tolerance for M. methanolica any further. The increments obtained by adaptation and mutation were easily achieved, but the temperature optimum did not exceed 37°C in any case. The stable isolate M13V, can very well be the result of a single mutation, although the mutagenic treatment doubtlessly had induced other changes as well, as will be reported in the following section. A further extension of the temperature range might require several mutations involving more than one complex mechanism. This is undoubtedly very difficult without simultaneously affecting of the organism in a negative direction, or without giving it characters not desirable from the production point of view. It is certainly an utmost delicate task to impose such extensive alterations, by means of simple mutagenic methods, in an already well functioning unity as the microbial cell. The few reports on such mutants illustrate clearly these difficulties.

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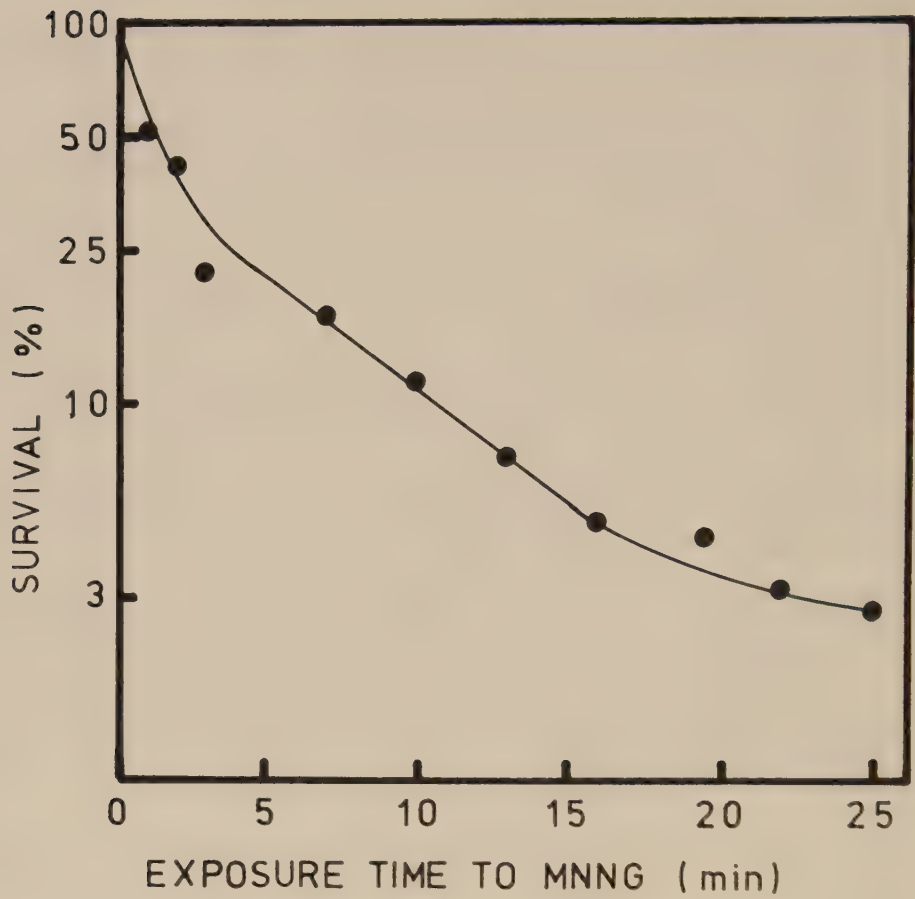


FIG 1. EFFECT OF MNNG ($100 \mu\text{g} \cdot \text{ML}^{-1}$) ON SURVIVAL OF METHYLOMONAS METHANOLICA AT 30°C IN A MINERAL SALTS SOLUTION OF PH 6.0.

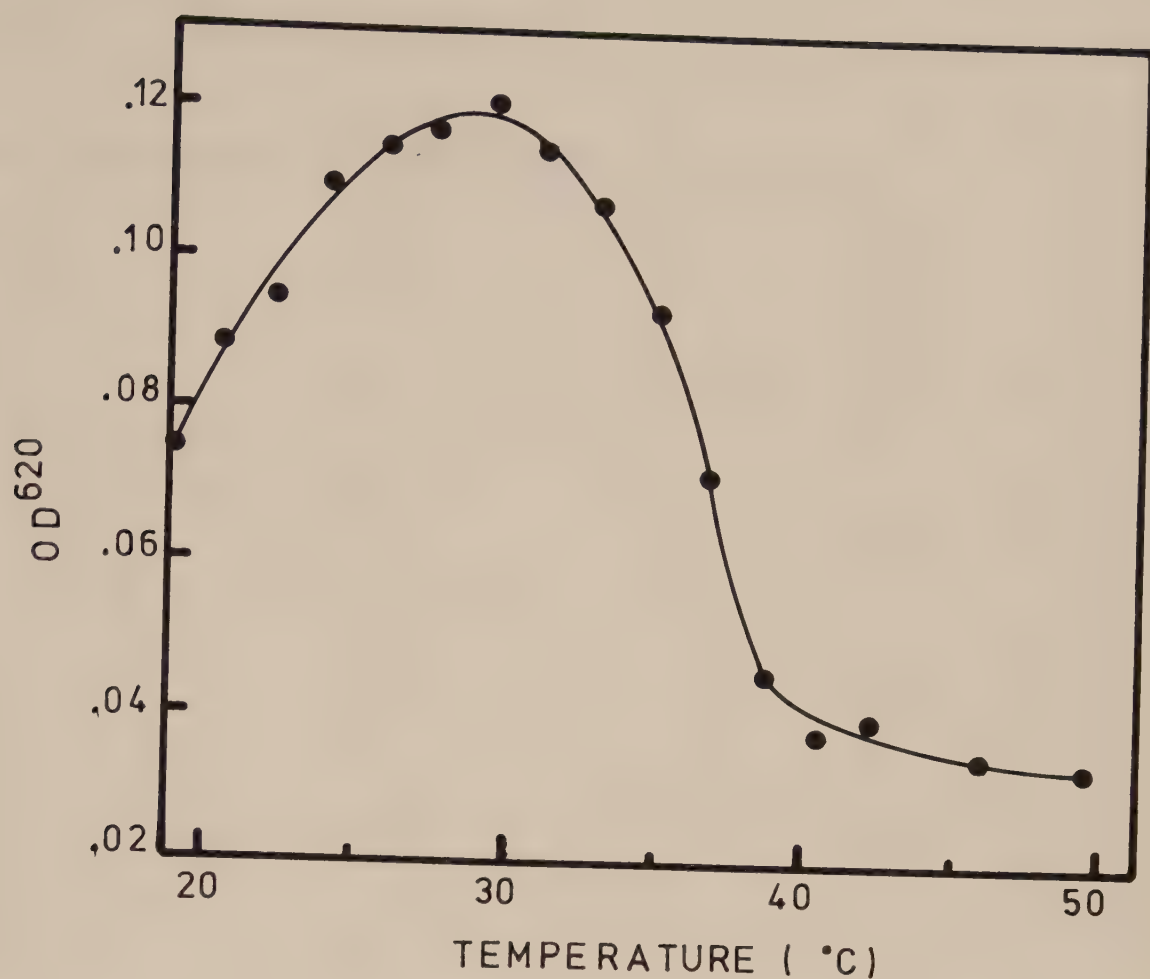


FIG 2. TEMPERATURE OPTIMUM CURVE FOR METHYLOMONAS
METANOLICA OBTAINED IN A TEMPERATURE GRADIENT
INCUBATOR. OPTICAL DENSITY WAS MEASURED IN
A LINSON 3 COLORIMETER.

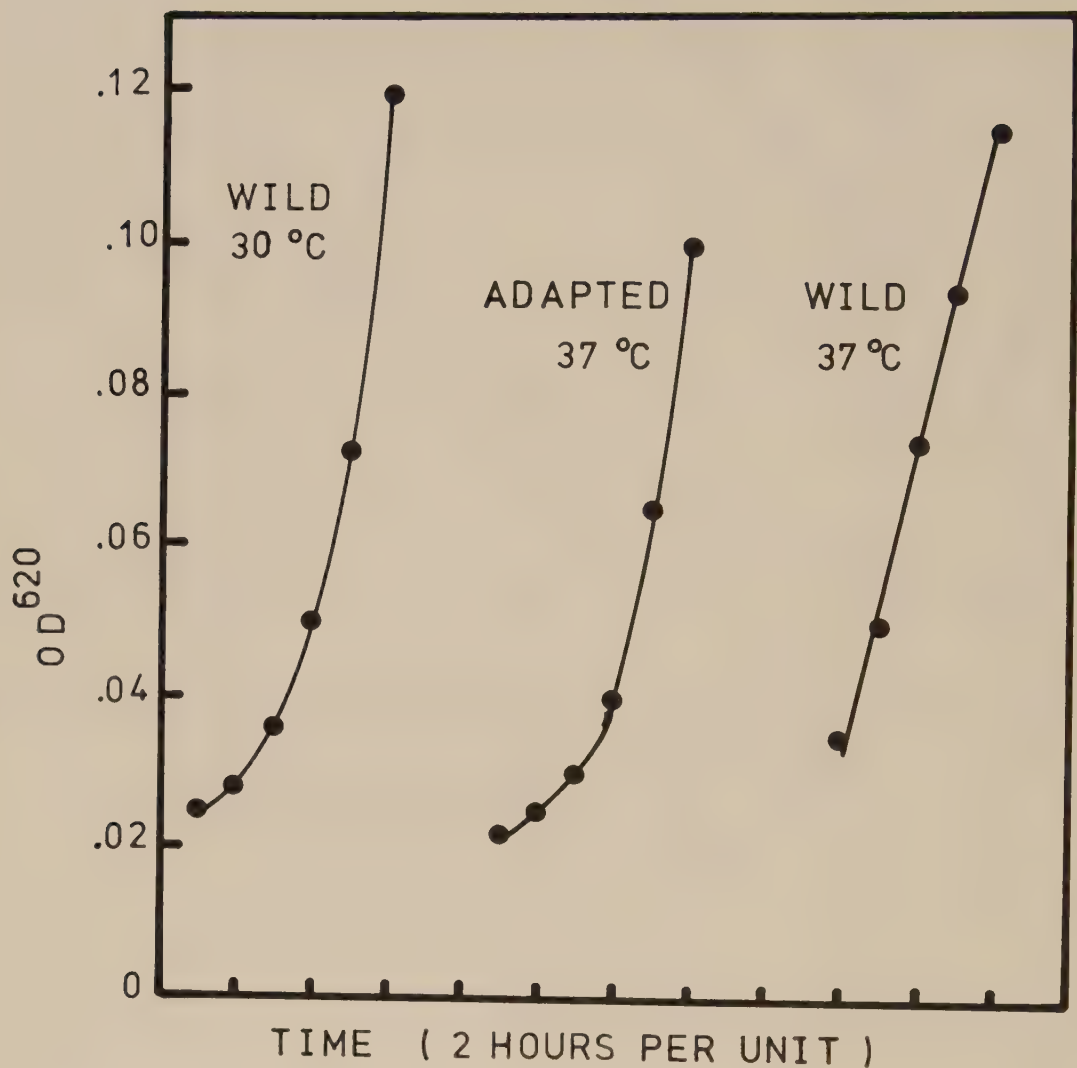


FIG 3. GROWTH CURVES FOR METHYLOMONAS METHANOLICA AND THE TEMPERATURE-ADAPTED CULTURE. DOUBLING TIME FOR THE WILD STRAIN AND THE ADAPTED CULTURE IS 1.6 H AT 30°C AND 37°C RESPECTIVELY. THE WILD STRAIN, DIRECTLY TRANSFERRED TO 37°C, HAS A LINEAR GROWTH.

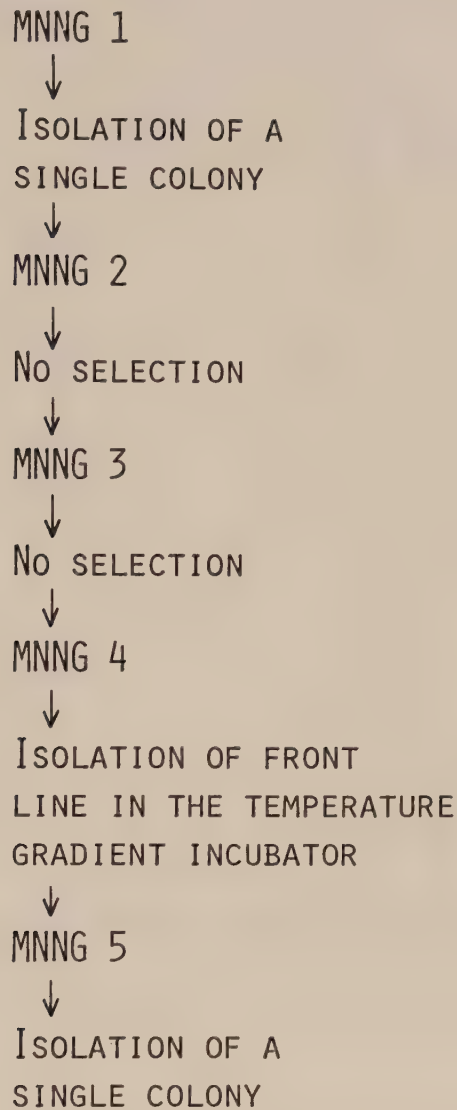


FIG 4. FLOW DIAGRAM OF MUTAGENIC TREATMENTS OF METHYLOMONAS METANOLICA IN ORDER TO OBTAIN TEMPERATURE TOLERANT VARIANTS.

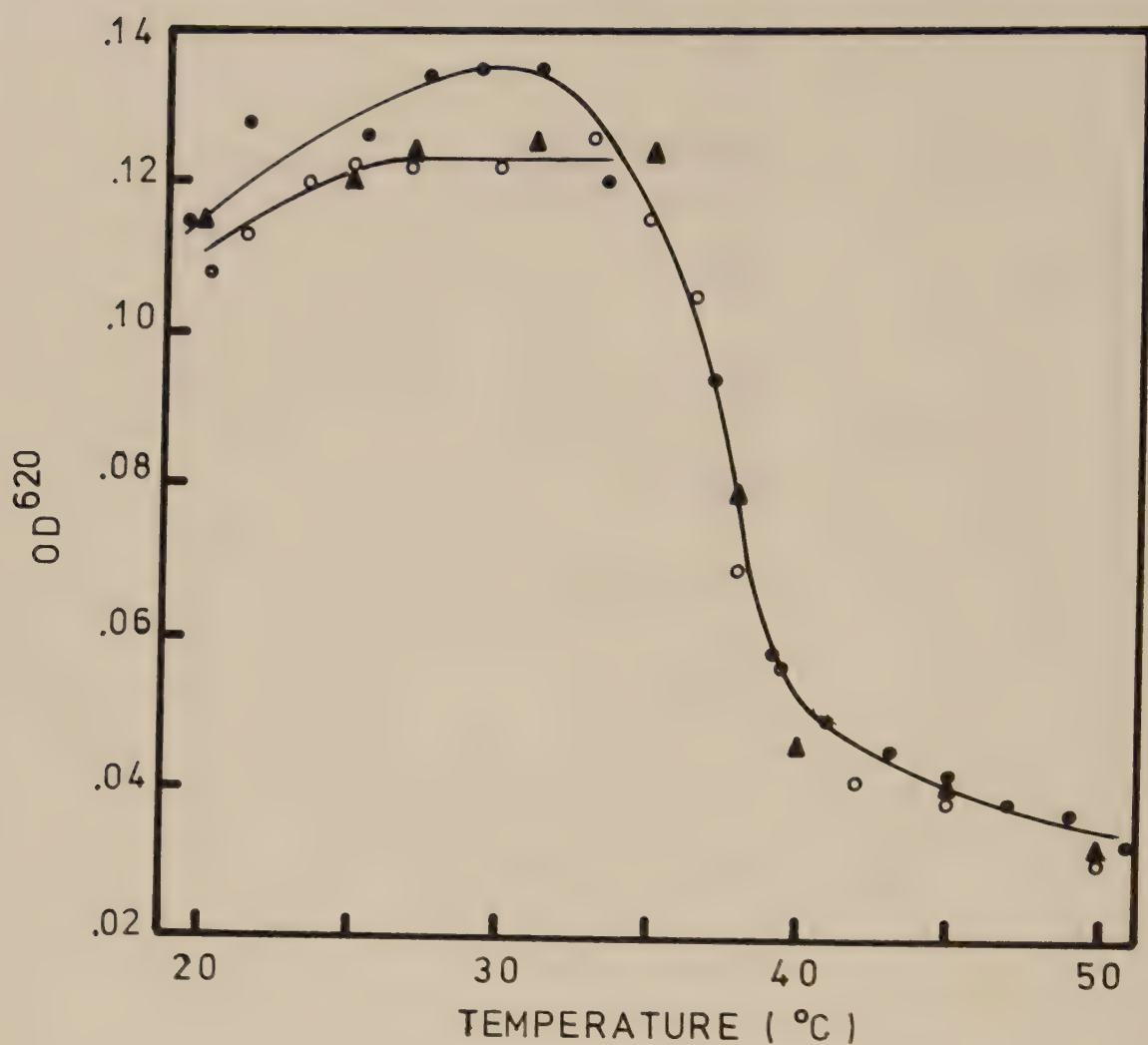


FIG 5. TEMPERATURE OPTIMUM CURVES FOR DIFFERENT ISOLATES AFTER ONE (—●—), FOUR (—○—), AND FIVE (—▲—) MUTAGENIC TREATMENTS OF METHYLOMONAS METHANOLICA. OPTICAL DENSITY WAS MEASURED IN A LINSON 3 COLORIMETER.

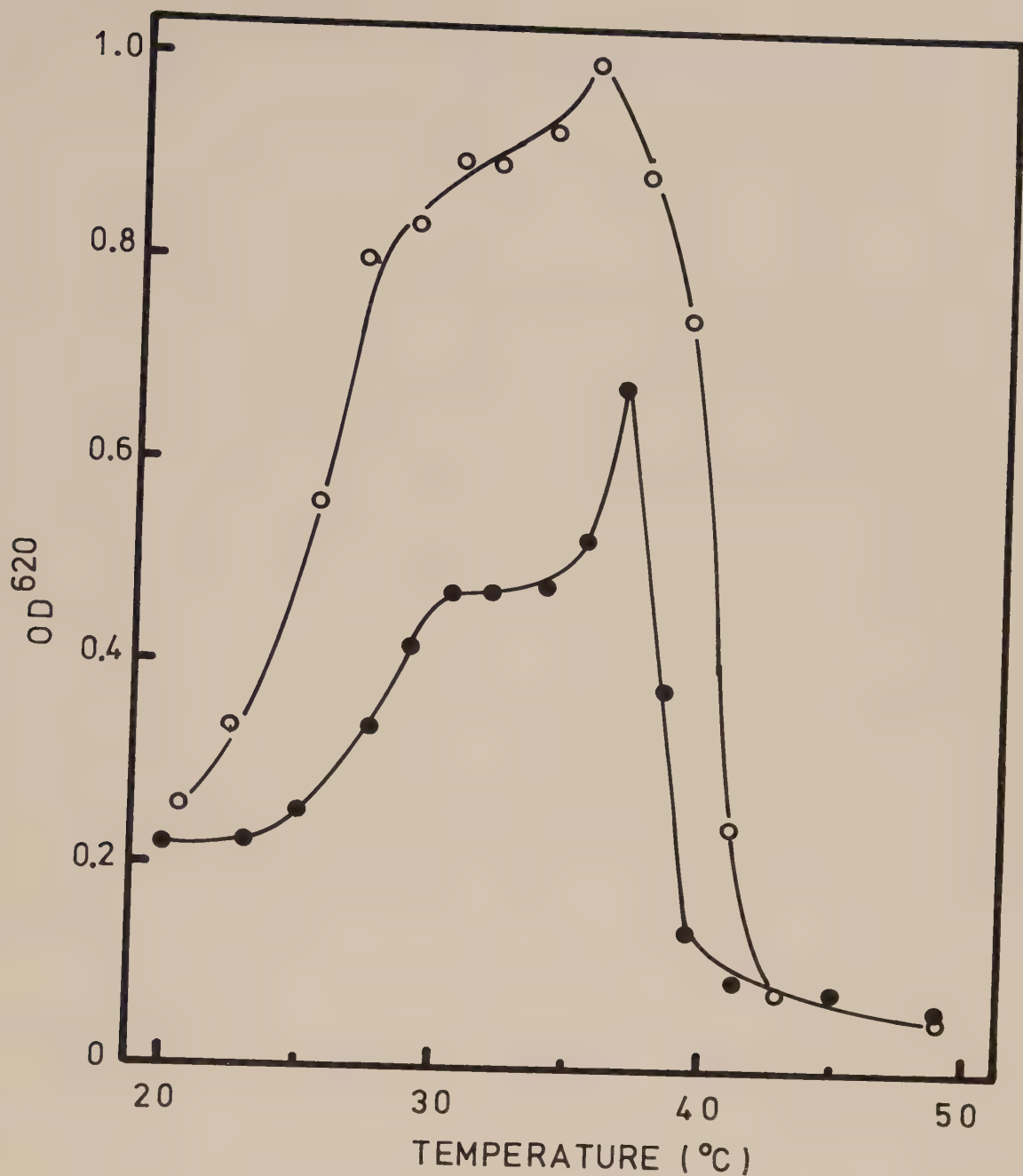


FIG 6. TEMPERATURE OPTIMUM CURVES FOR THE LAST ISOLATE AFTER MUTAGENIC TREATMENT. THE CULTURE HAD BEEN SUBJECTED TO ONE (—●—) AND THREE (—○—) CULTIVATIONS AT 40°C. OPTICAL DENSITY WAS MEASURED IN A TURNER COLORIMETER.

VI. COMPARISON OF METHYLOMONAS METHANOLICA AND THE MUTANT STRAIN M13V DERIVING FROM M. METHANOLICA

SUMMARY

Methylomonas methanolica and the mutant strain M13V are alike in basic features and have similar cell composition. The production of an extracellular slimy substance in strain M13V was discovered. This substance acted as a flocculant, causing sedimentation of both strains. At optimal temperature the thermotolerant mutant has the same maximum specific growth rate as the wild strain. Strain M13V had also acquired an extended resistance against elevated temperatures and low pH. The two strains were different in streptomycin resistance.

INTRODUCTION

The stable isolate, strain M13V, obtained after mutagenic treatment of Methylomonas methanolica has also attained characters other than the desired higher temperature tolerance. Some of these characters are relevant to its use in a process for production of SCP and some are not. The intention of this investigation was to characterize some of the differences between the mutant strain M13V and the wild strain M. methanolica from an applied aspect but also from a more fundamental point of view.

MATERIAL AND METHODS

Cultures

Methylomonas methanolica and strain M13V

Media

MM2 growth medium and MM2 agar medium,
W8 + W9 for fermenter cultivations.

Cultivation conditions

1. Shaking flasks 25-100 ml cultivation volume.
2. Biotec (4 l) fermentation unit or Chemoferm (4 l) fermentation unit equipped for batch culture.

Analyses

Optical density estimations (Turner)

Bacterial dry weight

Carbon, nitrogen and hydrogen in biomass

Metals and phosphorus in biomass

Anthrone test for carbohydrate (see section VII)

Survival at elevated temperatures

A shaken culture was centrifuged and resuspended in fresh MM2 salts solution. At time zero an appropriate amount of this suspension was added to 25 ml MM2 salts solution thermostated to the desired temperature, to obtain an OD value of 0.77.

1 ml samples were selected at intervals and immediately diluted 26 times in room temperature MM2 growth medium and then incubated at growth temperature for 24 hours. The same dilution procedure, except for heat treatment, was applied to a control sample. The number of viable organisms present in the sample after heat treatment would consequently grow during incubation, if sufficiently numerous but not if they were too few. After the incubation period the recovery from the heat treatment was measured by optical density. For comparison the quotient between OD of the sample and OD of the control was used.

Oxygen consumption

Oxygen consumption rates were measured with an oxygen electrode (see general methods) in a thermostated closed glass vessel with magnetic stirring. 9.5 ml of MM2 growth medium, saturated with air, was added to the system. Thereafter 0.5 ml of a concentrated bacterial culture, suspended in MM2 salts solution, was added to the reaction vessel. Oxygen consumption

was measured by the decrease in dissolved oxygen and continuously recorded. From known data on oxygen solubility in water at different temperatures, the oxygen saturation value of the liquid in equilibrium with air was calculated. The slope of the decreasing dissolved oxygen signal was then proportional to oxygen consumption rate. The records had an initial lag phase of a few seconds before dissolved oxygen began to decrease linearly. Below 30% dissolved oxygen the consumption rate was slower and this part was not used for calculations. If the cell suspension in the reaction vessel contained approximately $1 \text{ mg dry weight} \cdot \text{ml}^{-1}$ the whole experiment takes only 2-3 minutes at 30°C .

Assay for streptomycin resistance

Streptomycin sulphate (Str) was added at various concentrations to shaking flasks with MM2 growth medium. The flasks were inoculated and incubated at the appropriate temperature. Optical density was measured initially and then five times at one hour of interval and finally after 24 hours' incubation.

RESULTS

Some characteristics of *M. methanolica* and strain M13V

Colonies on agar plates exhibit different morphology in the two strains. M13V has larger, mucoid, dense colonies often appearing in clusters. Colonies of *M. methanolica* spread more regularly on plates, are smaller and nearly transparent. These characters were not invariable but subjected to changes as a result of incubation temperature. If strain M13V was incubated at 30°C the colonies became smaller and less mucoid and if *M. methanolica* was incubated at 37°C its colonies turned slightly larger and less transparent than usual. The mucoid appearance of the colonies originates from the production of an extracellular slimy substance.

The supernatant from liquid cultures of strain M13V, which became highly viscous at high cell densities, reacted positive

on the anthrone test thus proving the presence of a carbohydrate compound. The substance will henceforth be regarded as an extracellular polysaccharide. M. methanolica also produces an extracellular anthrone reactive material but at much lower concentrations. Liquid cultures of M. methanolica also does not become viscous in the same manner as for strain M13V. This phenomenon was reflected in the ratio between dry weight of cells and OD. At 35°C the dry weight for strain M13V is approximately 35% of the OD value and 50% for M. methanolica. At 30°C dry weight is 45% of the OD value for strain M13V, indicating a lower production of the polysaccharide at this temperature.

The mutant strain M13V has the ability to sediment spontaneously and to precipitate completely from the untreated cultivation medium on standing. M. methanolica shows only a very slight tendency to do so. Addition of supernatant from a mutant culture makes however, an otherwise untreated culture of the wild strain to sediment, when addition of mutant cells merely, do not cause any sedimentation. The mutant itself also fails to sediment if it is centrifuged and resuspended in fresh medium. Thus it is very likely that the polysaccharide in the medium causes the sedimentation, acting as a flocculating agent.

As noted earlier (section V) the mutant strain is more resistant against low pH than the wild strain. pH 8.5 is upper growth limit for both strains. M. methanolica can not grow at pH below 5.3 but the mutant still grows at pH 4 and is not totally inhibited until pH approaches 3.5. Thus, the mutant has obtained an extended range of pH tolerance.

The mutant strain M13V can like M. methanolica utilize methanol and methylamine as sole carbon sources. It requires (as does M. methanolica) an adaptation period before growth can commence on methylamine.

No significant difference in the elementary composition between the two strains were found (Table 1). The apparently higher content of calcium in the mutant may reflect natural variations.

Table 1. Elementary composition of strain M13V and Methylomonas methanolica.

Element		Strain M13V	M. methanolica
C	g/100 g dry matter	46.18	47.90
N	"	13.45	13.95
H	"	6.68	7.20
P	"	2.00	2.60
Mg	"	0.36	0.28
K	mg/100 g dry matter	290	346
Ca	"	305	191
Fe	"	96	115
Cu	"	57	33
Zn	"	14	10

Influence of temperature on growth rate, survival, and oxygen consumption

The maximum specific growth rate, $\mu_{\max}$, was measured in a number of fermenter batch cultivations at different temperatures (Fig.1). Optimum growth temperature for strain M13V was 35°C, a result in good agreement with the temperature optimum curves obtained earlier (section V). At 35°C and 40°C the mutant strain had higher $\mu_{\max}$ than the wild strain, but not at 30°C.

Since strain M13V grows at higher temperatures than M. methanolica one would expect that it exhibits a greater survival at temperatures above growth maximum. The experimental difficulties in proving this were however great. Heat killing is considered a first order kinetic function, which means that a comparison between two strains must be based on either a known number of organisms or otherwise on an equal number of organisms. Conventional viable count technique can not be used on these two strains. OD can otherwise be used as a measure for cell number, but this possibility is also ruled out as a

quantitative method, as the two strains have different quotients between dry weight and OD. In a preliminary experiment, using the method described in materials and methods, which in fact is based on OD measurements, it seemed however evident that the incubation temperature, prior to heat exposure, was essential for the survival. If the two strains were incubated at 37°C and then exposed to heat, both showed a greater resistance than when previously incubated at 30°C. Following this line both strains were grown at 37°C and exposed to 55°C still using the same method. The results (Fig.2) indicate that strain M13V has a slightly higher capability to recover after exposure to this temperature. This conclusion can be made without being too venturesome, since the initial cell number exposed to heat certainly was lower for strain M13V than for M. methanolica.

As a complement to these findings the oxygen consumption rate at different temperatures was investigated. If the oxygen consumption rate at 30°C is used as a standard for the microbial activity in the sample, then the quotient between consumption rate at a higher temperature and the consumption rate at 30°C will reflect the microbial activity at the higher temperature. Both strains had higher activity at 37°C than at 30°C which might reflect a simple Arrhenius function (Table 2). At 50°C the oxygen consumption rate was unchanged for M. methanolica when incubated at 30°C to this experiment but slightly lower when it previously was incubated at 37°C. For strain M13V the oxygen consumption rate had increased substantially for both the 30°C and the 37°C culture. It is thus clear that the mutant strain M13V has obtained an extended range of temperature tolerance.

Table 2. Influence of temperature on oxygen consumption rate for M. methanolica and strain M13V.

	Oxygen consumption rate					
	mg O ₂ ·min ⁻¹	Rel. act. _b	mg O ₂ ·min ⁻¹	Rel. act. _b	mg O ₂ ·min ⁻¹	Rel. act. _b
	30°C	30°C	37°C	37°C	50°C	50°C
M. methanolica						
(30°C) ^a	8.73	1.00	9.57	1.10	9.68	1.11
M. methanolica						
(37°C) ^a	4.90	1.00	5.20	1.06	4.31	0.88
Strain M13V						
(30°C) ^a	5.57	1.00	6.41	1.15	9.50	1.71
Strain M13V						
(37°C) ^a	4.67	1.00	5.20	1.11	7.96	1.70

a) Incubation temperature prior to experiment

b) The relative activity is the quotient between actual oxygen consumption rate and oxygen consumption rate at 30°C

Differences in streptomycin resistance

A clear difference in resistance against streptomycin was found between the wild strain and the mutant. Strain M13V was considerably more sensitive to the antibiotic. Growth is totally inhibited by 30 µg Str·ml⁻¹ and even 10 µg·ml⁻¹ has a marked influence on the growth rate at 37°C, the routine incubation temperature. M. methanolica can grow up to 300 µg Str·ml⁻¹ (30°C) although growth is affected at lower concentrations (Fig.3). Above a certain streptomycin concentration the final OD value in the sample was lower than for the control, but it was still possible to measure logarithmic growth over a period of time. It was also established that the incubation temperature was essential for the degree of resistance. If the mutant was incubated at 30°C prior to and under the exposure to

streptomycin, it could grow up to $50 \mu\text{g Str} \cdot \text{ml}^{-1}$, and if M. methanolica was incubated at 37°C it went correspondingly more sensitive thus being finally inhibited by approximately $200 \mu\text{g Str} \cdot \text{ml}^{-1}$ (Fig. 4).

DISCUSSION

Production of exopolysaccharides is a regular phenomenon in bacteria in general (7) and has also been reported for methane (4,8) and methanol (1,2) utilizing bacteria. Since it was shown that the wild strain also produced an anthrone reactive material, it is likely that the mutagenic treatment (section V) has merely amplified this property in strain M13V. The importance of the extracellular substance is in this context, however, on the sedimentation properties it gives to strain M13V and M. methanolica. Aggregation of microbial cells by means of either synthetic polymers or naturally produced, excreted or surface bound polymeric compounds is well documented (5). The polysaccharide production and sedimentation properties of strain M13V will be examined in detail in the following section.

Strain M13V had acquired an extended range of pH tolerance which ought to be an important factor in SCP production since it allows wider manipulations with the medium. The importance of a balanced medium has been demonstrated earlier (section IV). As a consequence of the special composition of this medium it was not really possible to use higher pH than 6 without precipitation as a result. It should also be beneficial if a production process could be run at lower pH thereby diminishing the contamination risk of other bacteria.

The mutant strain M13V showed not only a higher temperature optimum than M. methanolica but it had also retained the same maximum growth rate as the wild strain. This property had indeed been pursued. A high maximal growth rate is a priori valuable from a production point of view because it provides a wider range for possible cultivation conditions. Strain M13V was also more resistant against elevated temperatures than the wild strain was. This would be expected since it grows at higher temperatures.

It might be seen that investigation of antibiotic sensitivity is outside the scope of this report but covariation of resistance pattern to such antibiotics which interfere with the protein synthesis by binding to ribosomes e.g. streptomycin, and changes in temperature tolerance range has been reported. The temperature tolerant mutants of P. fluorescens (3) were all resistant to streptomycin in contrary to the sensitive wild strain. McDonald and Matney (6) has succeeded in transforming the ability to grow at 55°C from a thermophile Bacillus subtilis to a mesophilic strain of the same species. The recipient cells were from the beginning streptomycin resistant but 80-90% of the transformed cells had besides higher heat resistance simultaneously acquired streptomycin sensitivity from the donor cells. These results indicate a close coupling between the high temperature character and streptomycin locus. On the other hand no simple relationship seems to exist, as the high temperature character in one case is connected with streptomycin resistance and in the other case with streptomycin sensitivity.

The mutant strain M13V was considerably more sensitive to streptomycin than the wild strain. It was also shown that sensitivity increased with increasing incubation temperature for both the mutant and the wild strain. Regardless of incubation temperature however, the mutant was still more sensitive to the antibiotic. If there is a real functional relationship between the changes of antibiotic sensitivity pattern and the changes of temperature tolerance range, this would undoubtedly provide a short cut for the selection of temperature tolerant mutants.

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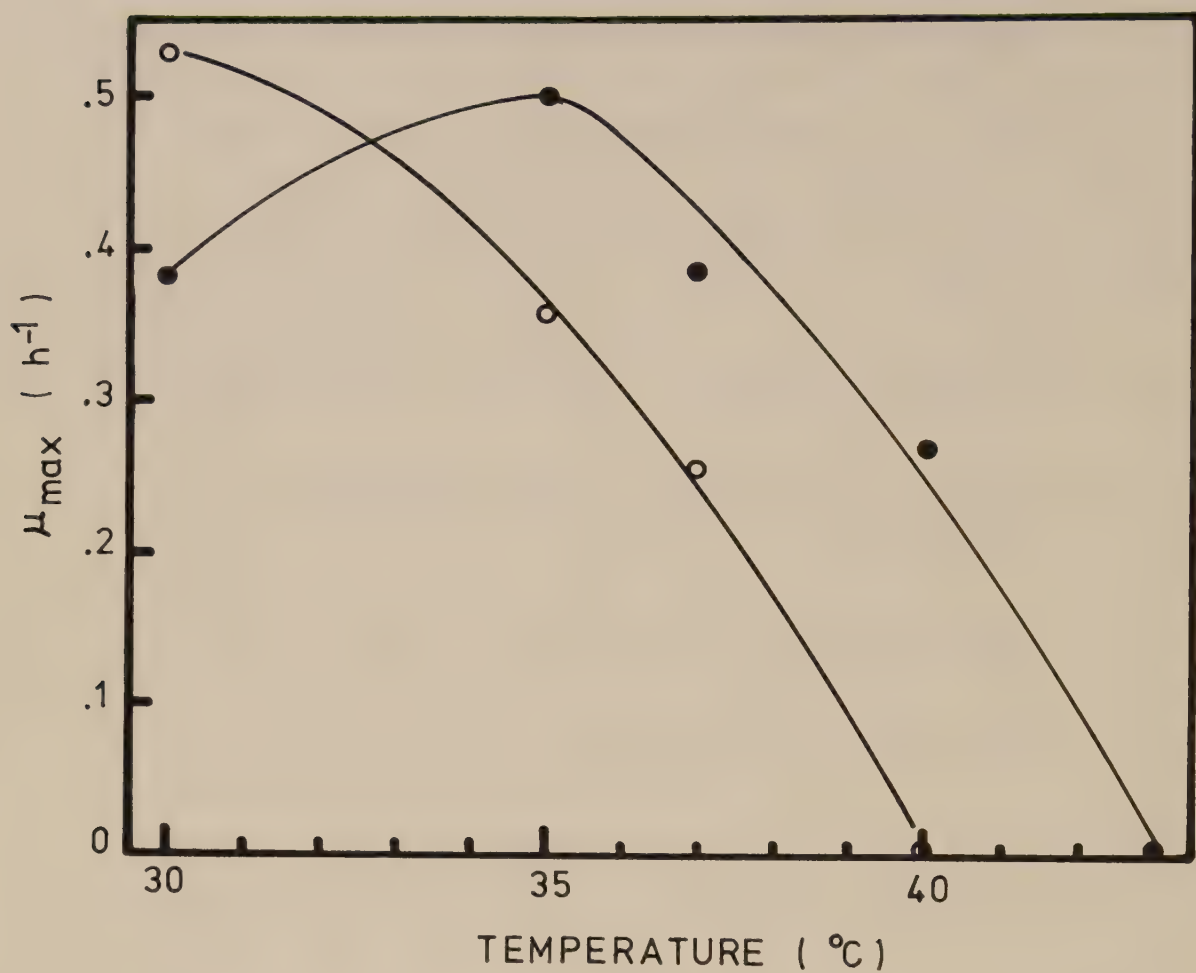


FIG 1. INFLUENCE OF TEMPERATURE ON MAXIMUM SPECIFIC GROWTH RATE ($\mu_{\max}$) FOR *METHYLOMONAS METAHNOLICA* (—○—) AND THE MUTANT STRAIN M13V (—●—).

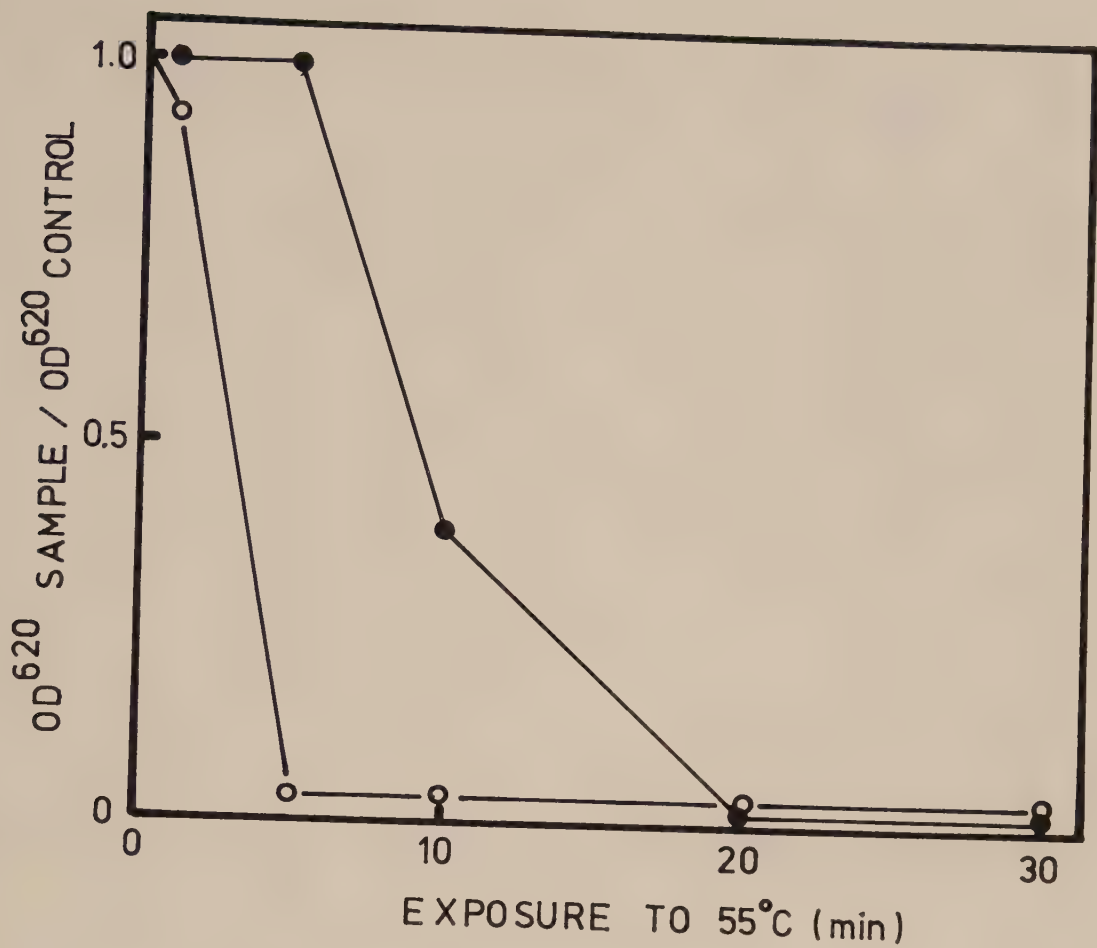


FIG 2. RECOVERY AFTER EXPOSURE TO 55°C FOR *METHYLOMONAS METHANOLICA* (—○—) AND STRAIN M13V (—●—), MEASURED AS THE QUOTIENT BETWEEN GROWTH AFTER HEAT-TREATMENT OF THE SAMPLE AND GROWTH IN A CONTROL WHICH WAS NOT HEAT-TREATED.

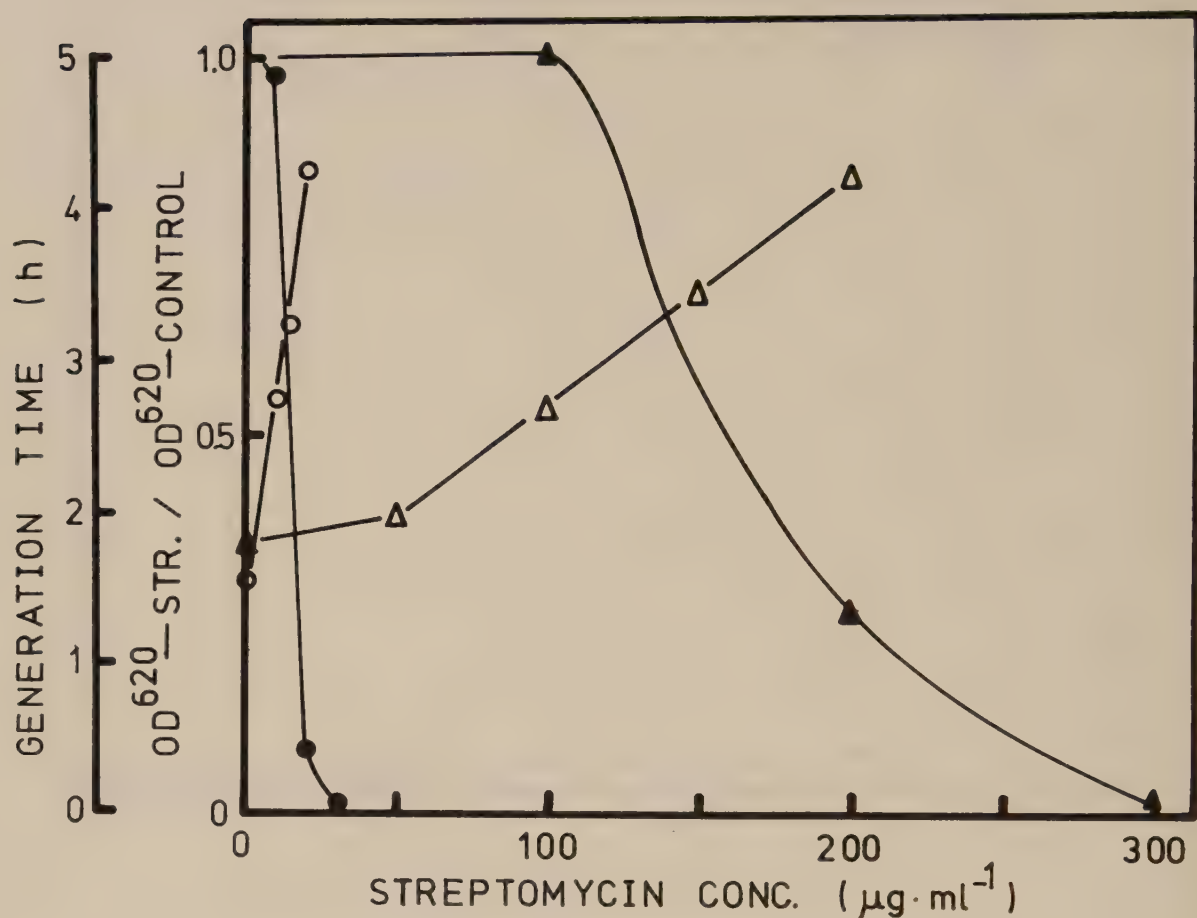


FIG 3. INFLUENCE OF STREPTOMYCIN ON GROTH OF METHYLOMONAS METHANOLICA AT 30°C AND STRAIN M13V AT 37°C. THE PARAMETERS MEASURED WERE GENERATION TIME (G) AND OPTICAL DENSITY EXPRESSED AS $\frac{OD \text{ Str.}}{OD \text{ Control}}$ (Q_{OD})

METHYLOMONAS METHANOLICA: G — Δ —
 Q_{OD} — ▲ —
 STRAIN M13V: G — ○ —
 Q_{OD} — ● —

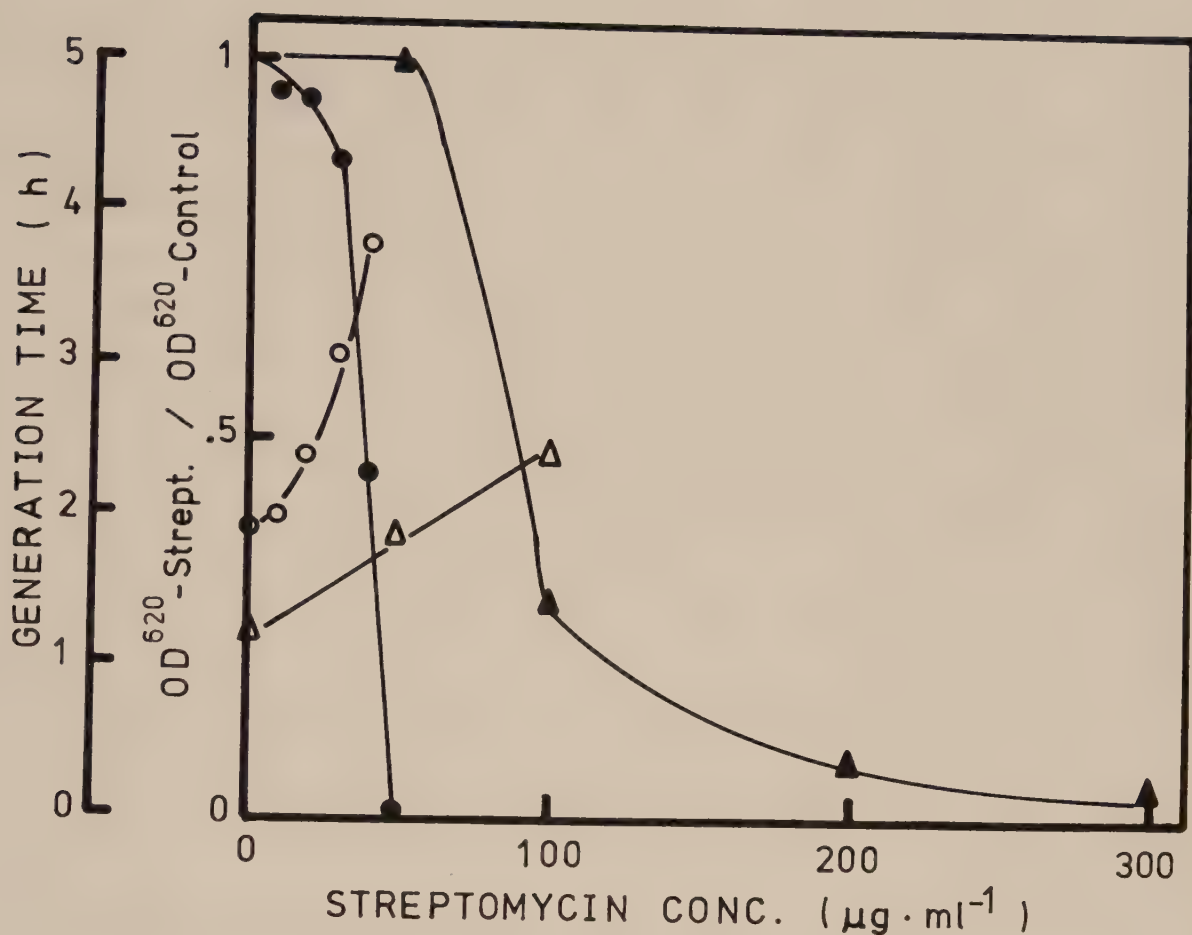


FIG 4. INFLUENCE OF STREPTOMYCIN ON GROWTH OF METHYLOMONAS METHANOLICA AT 37°C AND STRAIN M13V AT 30°C. THE PARAMETERS MEASURED WERE GENERATION TIME (G) AND OPTICAL DENSITY EXPRESSED AS $\frac{OD \text{ Str.}}{OD \text{ Control}}$ (Q_{OD}).

METHYLOMONAS METHANOLICA: G — Δ —
 Q_{OD} — ▲ —
 STRAIN M13V: G — ○ —
 Q_{OD} — ● —

VII. GROWTH CHARACTERISTICS OF THE METHANOL UTILIZING STRAIN M13V IN RESPECT TO BIOMASS PRODUCTION

SUMMARY

Strain M13V grown on methanol at 35°C has a maximum specific growth rate $\mu_{\max} = 0.50 \text{ h}^{-1}$. The yield coefficient for methanol is $0.39 \text{ g} \cdot \text{g}^{-1}$ and for oxygen $0.57 \text{ g} \cdot \text{g}^{-1}$. The carbon conversion factor for methanol was calculated to $0.48 \text{ g} \cdot \text{g}^{-1}$, the oxidation quotient to $0.68 \text{ mol} \cdot \text{mol}^{-1}$, and the respiratory quotient to $0.66 \text{ mol} \cdot \text{mol}^{-1}$. The production kinetics of an extracellular polysaccharide was analysed in batch and continuous culture. The exopolymeric substance makes strain M13V to sediment spontaneously. An optimal concentration was required for maximum sedimentation rate.

INTRODUCTION

A total evaluation of a microbial strain for SCP production involves many areas of different disciplines. In laboratory scale physiological characters are studied, followed by experiments in pilot plant scale including development of proper equipment and nutritional tests of the product. Results from such investigations are then used as a basis for an economical evaluation. The economical evaluation involves also costs for investments, substrate, cooling, aeration, separation, and treatment of waste water. A balance account between all known process parameters will finally decide if the process is economically feasible and also the most favourable conditions for its realization.

The most important factors regarding the microorganism itself are: growth rate, productivity, yield on carbon source, oxygen demand, temperature range for growth, harvesting properties and nutritional value. This section will deal with these properties in strain M13V cultivated in laboratory fermenters, leaving the nutritional aspects aside. The growth characteristics of strain M13V will be discussed in physiological terms as well as in relation to SCP production.

MATERIALS AND METHODS

Culture

Strain M13V

Medium

W8 + W9 for fermenter cultivations

Cultivation conditions

1. Batch cultures in Biotec (4 l) fermentation unit or Chemoferm (4 l) fermentation unit.
2. Continuous cultures in Biotec (4 l) fermentation unit or Chemoferm (4 l) fermentation unit. The chemostat principle for continuous culture was applied using methanol as the limiting substrate. At steady state conditions is the dilution rate equal to the growth rate (μ). These expressions are used synonymously in the text.

Analyses

Bacterial dry weight

Methanol in flow medium for continuous culture

Anthrone analysis

Quantitative estimation of polysaccharide formation of strain M13V was performed according to the method described by Scott and Melvin (14). The anthrone test reacts on total carbohydrate present in the sample and concentrations of the test compound are expressed in equivalents to the glucose standard.

Gas analyses

Oxygen and carbon dioxide were measured in the outgoing air-stream from the continuous culture. Samples were periodically injected in a Varian gas chromatograph model 920 via an 1 ml sampling loop. The gas chromatograph was equipped with

thermistor detector and two columns in series, one 6' x 1/8" column with Porapak Q at 80°C for separation of CO₂ and one 6' x 1/8" column with Molecular Sieve 5A at room temperature for separation of oxygen and nitrogen. The second column was connected between the sample detector and the reference detector. For calibration, standard gas with 5% carbondioxide and 20% oxygen was used (AGA, Lidingö, Sweden). Calculations of oxygen uptake and carbon dioxide production were based on the formula by Fiechter and Meyenburg (11), which requires data on oxygen and carbon dioxide in ingoing and outgoing airstream, flow of ingoing airstream and cultivation volume.

Sedimentation

The sedimentation rate of strain M13V was analysed directly in untreated samples from a batch culture. 5.0 ml of the culture was transferred to a standard test tube and placed in a Beckman model C colorimeter with red filter. Optical density was continuously recorded by a single channel recorder. The photocell was placed 2 cm from the bottom of the test tube so the sediment would not interfere with OD readings. As the cells began to sediment, a record on decreasing OD against time was obtained. Since the initial OD for the sample always was higher than what could be directly measured, the sedimentation record started at that moment the OD fell within the resolution power of the instrument.

This means that the record describes the last sedimentation front passing the photocell. Sedimentation was almost complete. The final OD value was usually 0.2 which should be compared to initial values of 10-20. The records showed however some inconsistencies at low OD, why the time required for OD to decrease from 1.3 to 0.7 was taken as a measure on the sedimentation rate.

RESULTS

Batch culture

During the initial growth studies in batch culture it became evident that oxygen was a critical factor in biomass production from strain M13V. The oxygen transfer rate (OTR) is known to decrease with increasing viscosity of the liquid. The high viscosity developing in cultures of strain M13V therefore decreases the actual OTR to a much lower level than can be predicted from the OTR values, measured by the sulfite oxidation method. Fig.1 shows the influence of OTR (sulfite oxidation values) on dissolved oxygen and biomass production at 35°C. At approximately a duplicated increase of the OTR, biomass production increases twice at the level of zero % dissolved oxygen. Maximum productivity in batch culture at 35°C under standard cultivation conditions (see general methods) was $2.05 \text{ g} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ and the final biomass concentration was $11.0 \text{ g} \cdot \text{l}^{-1}$. Growth was logarithmic to $3.0 \text{ g} \cdot \text{l}^{-1}$ with a productivity of $1.50 \text{ g} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ at the end of the logarithmic growth phase (Fig.2).

Production of an extracellular polysaccharide in batch culture

The mutant strain M13V produces an extracellular slimy substance at first observed from the mucoid colony appearance and by the high viscosity developing in batch cultures. The substance was analysed according to the anthrone method thus measuring total carbohydrate, expressed as glucose equivalents, in the supernatant from batch cultures. A typical batch course at 35°C is shown in Fig.3. As much as $2 \text{ g} \cdot \text{l}^{-1}$ (glucose equivalents) of the compound was found at a cell density of $11 \text{ g} \cdot \text{l}^{-1}$. In Fig.4 are the product concentration (P), the production rate ($\frac{dP}{dt}$), and the specific rate of product formation ($\frac{dP}{dt} \cdot \frac{1}{X}$) plotted against biomass (X). In Fig.5 are the specific rate of product formation, the production rate, and the product yield (Y_P , g product produced/g biomass) shown as a function of $\frac{1}{X}$ growth rate. These curves describes mainly polysaccharide production in the declining batch phase. The logarithmic growth phase ends at a biomass concentration of

3 g.l^{-1} (Fig.4) which corresponds to a growth rate of 0.45 h^{-1} (Fig.5). The specific rate of product formation reached its maximum in the late logarithmic phase and decreased gradually in the declining phase. The total production rate and product yield increased however throughout the declining phase and the maximum amount of product accumulated not until growth had practically ceased. Similar results have been reported for polysaccharide production in other species (10). The same pattern for product formation was found in batch cultures at 30°C and 37°C . Product concentration and production rate varied however with the temperature. The synthesis of the polysaccharide increased evidently with increasing temperature (Table 1).

Table 1. Influence of temperature on the production of the extracellular polysaccharide in strain M13V.

Temperature	30°C	35°C	37°C
Product conc. (g.l^{-1}) at $3.5 \text{ g biomass.l}^{-1}$	0.21	0.37	0.52
Production rate ($\text{g.l}^{-1}.\text{h}^{-1}$) at $3.5 \text{ g biomass.l}^{-1}$	0.10	0.15	0.19

The mutant strain M13V has the ability to sediment spontaneously. This character was caused by the extracellular polysaccharide (section VI). The concentration of this factor proved to be essential. The sedimentation rate, measured as described in materials and methods, increased with increasing biomass and thereby with increasing concentrations of the product (Fig.6), until it reached a maximum level. Biomass in itself was not critical for the sedimentation rate, since dilutions of the cells in its own supernatant did not affect the sedimentation rate unless at very low biomass concentrations.

Continuous culture

Growth and productivity in a methanol limited chemostat followed perfectly the results predictable from the batch culture experiments. Maximum productivity obtained in continuous culture was $1.56 \text{ g} \cdot \text{l}^{-1} \cdot \text{h}^{-1}$ at a biomass concentration of $3.57 \text{ g} \cdot \text{l}^{-1}$ and a dilution rate of 0.435 h^{-1} (Fig.7). At this point the level of dissolved oxygen was 10% of full saturation. A further increase in biomass at this dilution rate would have made the culture oxygen limited.

The yield coefficient for methanol $\frac{Y_x}{s}$ (g biomass produced/g methanol consumed) was determined at 30°C , 35°C and 37°C in continuous culture. $\frac{Y_x}{s}$ was measured as the effective yield coefficient i.e. $\frac{Y_x}{s}$ calculated as the biomass yield on methanol in inflowing medium. Any correction for residual methanol in the chemostat would thus increase it. Maximum yield was found at 35°C . At 30°C and 37°C the conversion of methanol was less effective. Table 2 summarizes these figures together with the corresponding values on maximum specific growth rate (μ_{max}).

Table 2. Influence of temperature on the maximum specific growth rate (μ_{max}) and the yield coefficient for methanol ($\frac{Y_x}{s}$) for strain M13V.

Temperature ($^\circ\text{C}$)	μ_{max} (h^{-1})	$\frac{Y_x}{s}$ ($\text{g} \cdot \text{g}^{-1}$)
30	0.38	0.28
35	0.50	0.39
37	0.39	0.33
40	0.27	-

The yield coefficient for methanol was further determined at different dilution rates at 35°C . The effective $\frac{Y_x}{s}$ thus

measured was found to be independent of the growth rate with a mean value of $0.39 \text{ g} \cdot \text{g}^{-1}$ (Fig.8). The yield coefficient for oxygen Y_{O_2} ($\text{g } O_2 \text{ consumed/g biomass produced}$) and the carbon dioxide W_{CO_2} production coefficient ($\text{g } CO_2 \text{ produced/g biomass}$) were also determined at steady state conditions at different dilution rates at 35°C (Fig.8). Oxygen conversion efficiency increased with increasing growth rate while carbon dioxide production decreased with increasing growth rate. As a consequence of these relationships the carbon conversion factor ($C_{\text{biomass}}/C_{\text{methanol}}$) (calculations based on an average carbon content of 46.18%) was constant while the oxidation quotient OQ ($\text{mol } O_2 \text{ consumed/mol } CH_3OH \text{ consumed}$) decreased and the respiratory quotient RQ ($\text{mol } CO_2 \text{ produced/mol } O_2 \text{ consumed}$) increased slightly with increasing growth rates (Fig.9). Table 3 reviews the growth characteristics of strain M13V.

Table 3. Growth characteristics of strain M13V at 35°C . Most favourable values.

μ_{max}	0.50 (h^{-1}) maximum specific growth rate
$Y_{\underline{x}}$	0.39 ($\text{g} \cdot \text{g}^{-1}$) yield coefficient for methanol
$Y_{O_2}^s$	0.57 ($\text{g} \cdot \text{g}^{-1}$) yield coefficient for oxygen
W_{CO_2}	1.58 ($\text{g} \cdot \text{g}^{-1}$) production coefficient for carbon dioxide
$\frac{C_{\text{biomass}}}{C_{\text{methanol}}}$	0.48 ($\text{g} \cdot \text{g}^{-1}$) conversion factor for methanol carbon
O.Q.	0.68 ($\text{mol} \cdot \text{mol}^{-1}$) oxidation quotient
R.Q.	0.66 ($\text{mol} \cdot \text{mol}^{-1}$) respiratory quotient

The production of the extracellular polysaccharide in continuous culture was measured in the same way as for batch cultures. At steady state conditions the product yield $Y_{\underline{P}}$ was found to decrease with increasing dilution rates while the $\frac{Y_{\underline{P}}}{\underline{X}}$ specific rate of product formation ($\frac{dP}{dt} \cdot \frac{1}{\underline{X}}$) increased (Fig.10).

DISCUSSION

No quantitative data for polysaccharide production in methanol utilizing bacteria have been published earlier. The results reported here were obtained under the standard cultivation conditions, but the polysaccharide production might very well be susceptible to changes in the environment other than the temperature. Extracellular polysaccharide production is usually favoured by nutrient limitation, especially nitrogen or phosphorus limitation, in the presence of excess carbon and energy source (10,17) thereby providing a high C:N ratio. Other nutrients e.g. Ca^{2+} and Mg^{2+} are reported to be required or to stimulate the polysaccharide production (5). Oxygen is usually required for maximal polymer production (10) although this is not always the case (9).

The kinetics of product formation is usually expressed in one of three models.

a) Growth associated production

$$\frac{dP}{dt} = \alpha \cdot \frac{dX}{dt} \quad \text{or} \quad \frac{dP}{dt} \cdot \frac{1}{X} = \alpha \cdot \mu$$

b) Non growth associated production

$$\frac{dP}{dt} = \beta \cdot X \quad \text{or} \quad \frac{dP}{dt} \cdot \frac{1}{X} = \beta$$

c) Partly growth associated production

$$\frac{dP}{dt} \cdot \frac{1}{X} = \alpha \cdot \mu + \beta$$

The first model implies that the specific rate of product formation is directly proportional to the growth rate with a proportionality constant α . The second model that it is constant at a rate β independent of growth rate and biomass concentration. In the third model is the specific rate of product formation partly growth associated and partly independent of growth rate.

In continuous culture the kinetics of polysaccharide formation followed the third of the models above. Since $\frac{dP}{dt} \cdot \frac{1}{X} = Y_P \cdot \mu$

follows that $\frac{Y_P}{\bar{X}} \cdot \mu = \alpha \cdot \mu + \beta$ or $\frac{Y_P}{\bar{X}} = \alpha + \beta \cdot \frac{1}{\mu}$ (d).

In Fig.10 is $\frac{Y_P}{\bar{X}}$ shown as a function of μ . If linear regression analysis is $\frac{Y_P}{\bar{X}}$ applied to the experimental data, $\frac{Y_P}{\bar{X}}$ is found to be a linear function of $\frac{1}{\mu}$ with a regression coefficient of 0.99. α is the limes value for $\frac{Y_P}{\bar{X}}$ when μ approaches infinity and β is the slope of the line described by equation (d). α was calculated to 0.042 ($g \cdot g^{-1}$) and β to 0.011 (h^{-1}). Thus, for polysaccharide production in strain M13V in a methanol limited chemostat at 35°C the following equation is valid:

$$\frac{dP}{dt} \cdot \frac{1}{\bar{X}} = 0.042 \cdot \mu + 0.011.$$

The kinetics of polysaccharide production in batch culture follows essentially the same laws as in continuous culture but is more complex. The product yield ($\frac{Y_P}{\bar{X}}$) increases at low growth rates whereas the specific $\frac{Y_P}{\bar{X}}$ rate of product formation decreases at the beginning of the declining phase but increases slightly again at the end of it (Fig.5). The values for specific rate of product formation and product yield were however higher in the declining batch phase than in continuous culture, e.g. at $\mu = 0.25 h^{-1}$ is $\frac{Y_P}{\bar{X}}$ 0.19 in batch culture and 0.09 in continuous culture. In batch culture is the growth limiting factor in the declining phase mainly oxygen with an excess of carbon and nitrogen. In continuous culture the carbon source was used as the limiting factor whereas oxygen and nitrogen were in excess. These results indicate that high polymer production is favoured by low growth rate and possibly also by oxygen limitation in excess of carbon source.

The sedimentation rate of strain M13V was shown to be dependent on the polysaccharide concentration. If synthetic (2) or naturally produced (6) polymers are used as flocculating agents, aggregation increases with increasing concentrations of the flocculants, reaching a maximal level at the optimal concentration, whereafter it decreases again. The same conditions has been found in strain M13V since the sedimentation

rate increased with increasing concentrations of the exopolymeric substance and reached a maximum value. Higher polysaccharide concentrations would have been necessary to reflect if any decrease in sedimentation rate would occur. In a production process where harvesting of the small bacterial cells is one of the big problems both economically and technically (12,16), it should be an obvious advantage if a strain with aggregating properties could be used.

Production of SCP in a commercial scale is always carried out as a carbon source limited chemostat, because this is the most economical way to utilize the substrate. As a rule, a growth rate somewhere around the half of the maximum specific growth rate is chosen. Higher growth rates will, according to Michaelis-Menten kinetics, increase the residual carbon source concentration to too high levels. Lower growth rate will on the other hand decrease residual substrate but has a dramatic effect on total operating costs (1). For these reasons this investigation was restricted to studies in the range of $\mu = 0.15 - 0.4 \cdot h^{-1}$, which sufficiently covers the actual region for strain M13V at 35°C.

The maximum value on $\frac{Y}{X}$ for strain M13V was found at 35°C, the optimal growth temperature. The yield coefficient for methanol was independent of the growth rate but oxygen demand and carbon dioxide production decreased at higher growth rates. The two latter features can possibly be explained in terms of a more effective energy metabolism at high growth rates. The decrease in polysaccharide production at high growth rates, should mean that less energy is required per g of biomass, as apparently is reflected in the decreased carbon dioxide production. On a molar basis, the oxygen demand decreased more drastically than the carbon dioxide production as revealed by an increased respiratory quotient. This can be explained partly by less oxygen required for incorporation in the product and partly by the lower energy requirement. One would thus expect that the conversion efficiency of methanol also was affected at high growth rates. Correction for residual methanol in the chemostat would not affect the

experimental data appreciately since occasional measurements showed that outgoing methanol was at the most about 1% of input methanol concentration. The constant $Y_{\frac{x}{s}}$ results in fact in a decreasing cellular yield since less organic material is produced totally from the same amount of methanol. This can of course be the result if the maintenance energy required increases with the growth rate, but would on the contrary result in increased carbon dioxide production. It is also possible that the increased specific rate of polysaccharide formation requires more energy, thus counter balancing the lower product yield. Up to this point it has been assumed that the biomass has a constant composition. The carbon conversion factor was also calculated on basis of an average carbon content of the cells. If the cell composition changes with the growth rate the yield coefficient for methanol would still be the same but the carbon conversion factor would change. This could then give an indication on the carbon flow from methanol to biomass, carbon dioxide and product. But it is certainly not possible to fully explain these relationships without knowledge about the stoichiometry and energy requirements for the product formation.

The effective $Y_{\frac{x}{s}}$ for strain M13V is a rather normal value for methanol $\frac{x}{s}$ utilizers. The theoretical maximum value has been calculated to $0.73 \text{ g} \cdot \text{g}^{-1}$ (7) but experimental data reported in the literature are in the range of $0.3\text{-}0.5 \text{ g} \cdot \text{g}^{-1}$ (3,8,13,15) (see also section I and IV). In strain M13V is $Y_{\frac{x}{s}}$ independent of the growth rate in contrary to other findings (8,15), where the maximum value is obtained only at very high growth rates. This makes it possible to utilize the whole growth range for strain M13V if desired, without losing in conversion efficiency even at low growth rates.

The maximum theoretical yield coefficient for oxygen has been calculated to $0.7 \text{ g} \cdot \text{g}^{-1}$ (4) for methanol utilizing bacteria. Reviewing the literature however, there is only one report on experimental data. In the extensive studies on Methylomonas methanolica by Dostálek and Molin (8) the maximum Y_{O_2} was calculated to $0.53 \text{ g} \cdot \text{g}^{-1}$ which should be compared to $0.57 \text{ g} \cdot \text{g}^{-1}$ for strain M13V.

Summarizing the characters of strain M13V from a production aspect one can conclude that its growth characteristics i.e. high growth rate at 35°C, favourable values on $Y_{\frac{x}{s}}$ and Y_{O_2} in combination with a wide pH range and the extremely valuable sedimentation property, makes it attractive as a production strain. On the negative side is a rather low productivity which is caused by oxygen limitation due to the viscosity created by the polysaccharide. There are however, good reasons to believe that the production of this substance can be manipulated in such a way that a balance between its negative and positive effects can be achieved. If conditions for minimal polysaccharide production could be defined, it is easy to visualize a two step continuous culture where biomass is produced in the first fermenter and the culture is allowed to produce the sedimentation causing polysaccharide in the second.

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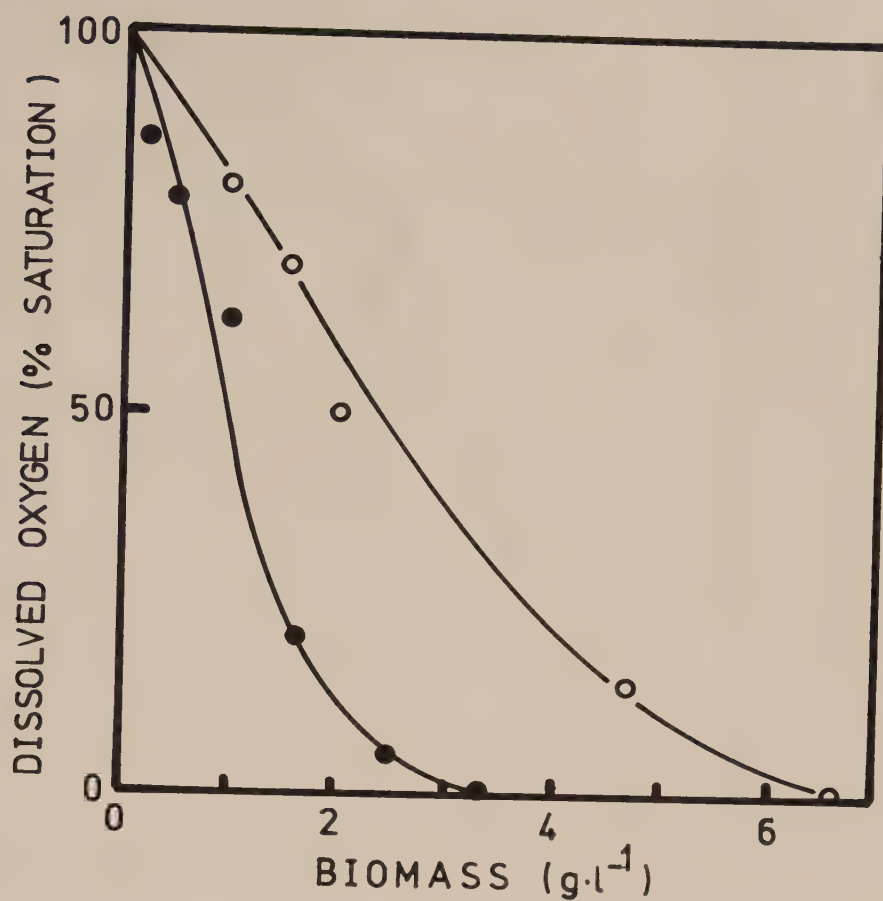


FIG 1. INFLUENCE OF OXYGEN TRANSFER RATE (OTR) (SULFITE OXIDATION VALUES) ON DISSOLVED OXYGEN AND BIOMASS PRODUCTION IN BATCH CULTURES OF STRAIN M13V AT 35°C.

OTR = 230 M MOL·L⁻¹·H⁻¹ (—●—)

OTR = 400 M MOL·L⁻¹·H⁻¹ (—○—)

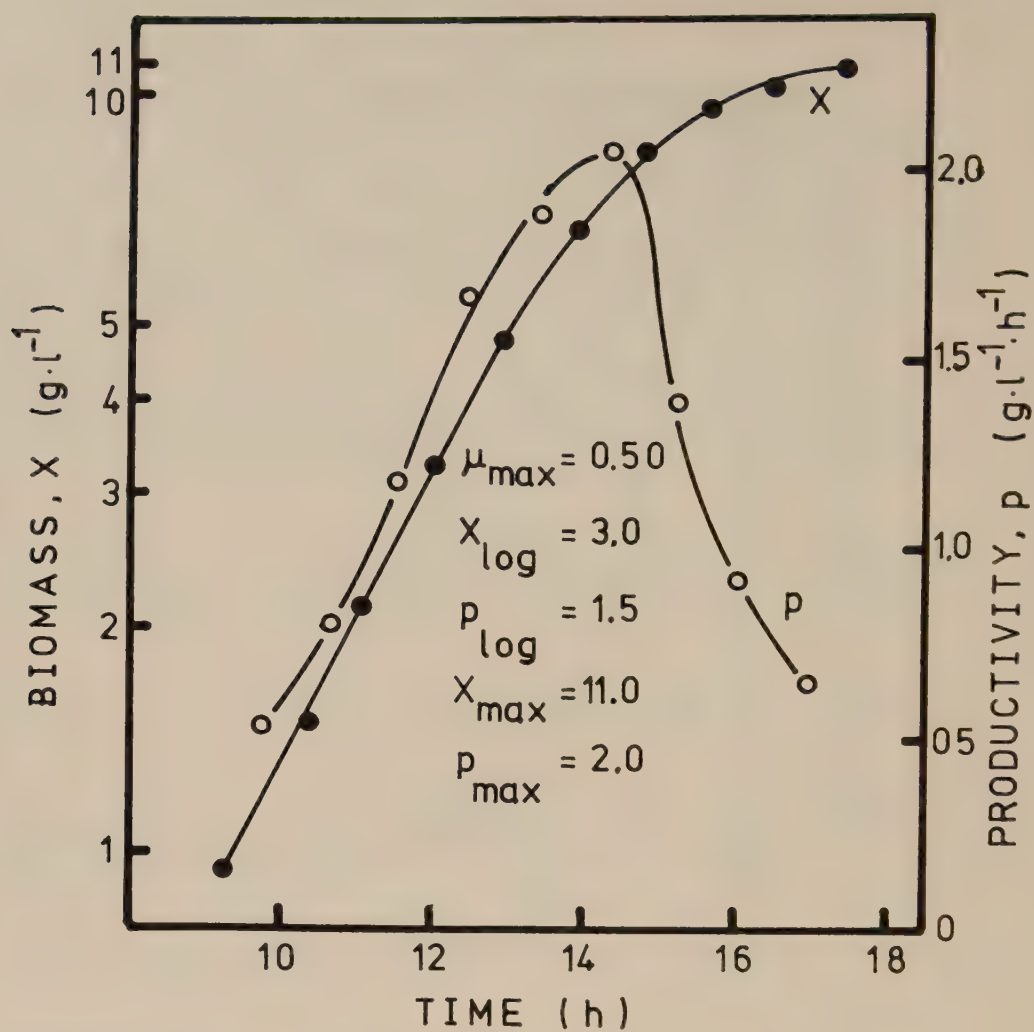


FIG 2. GROWTH AND PRODUCTIVITY FOR STRAIN M13V IN BATCH CULTURE AT 35°C .

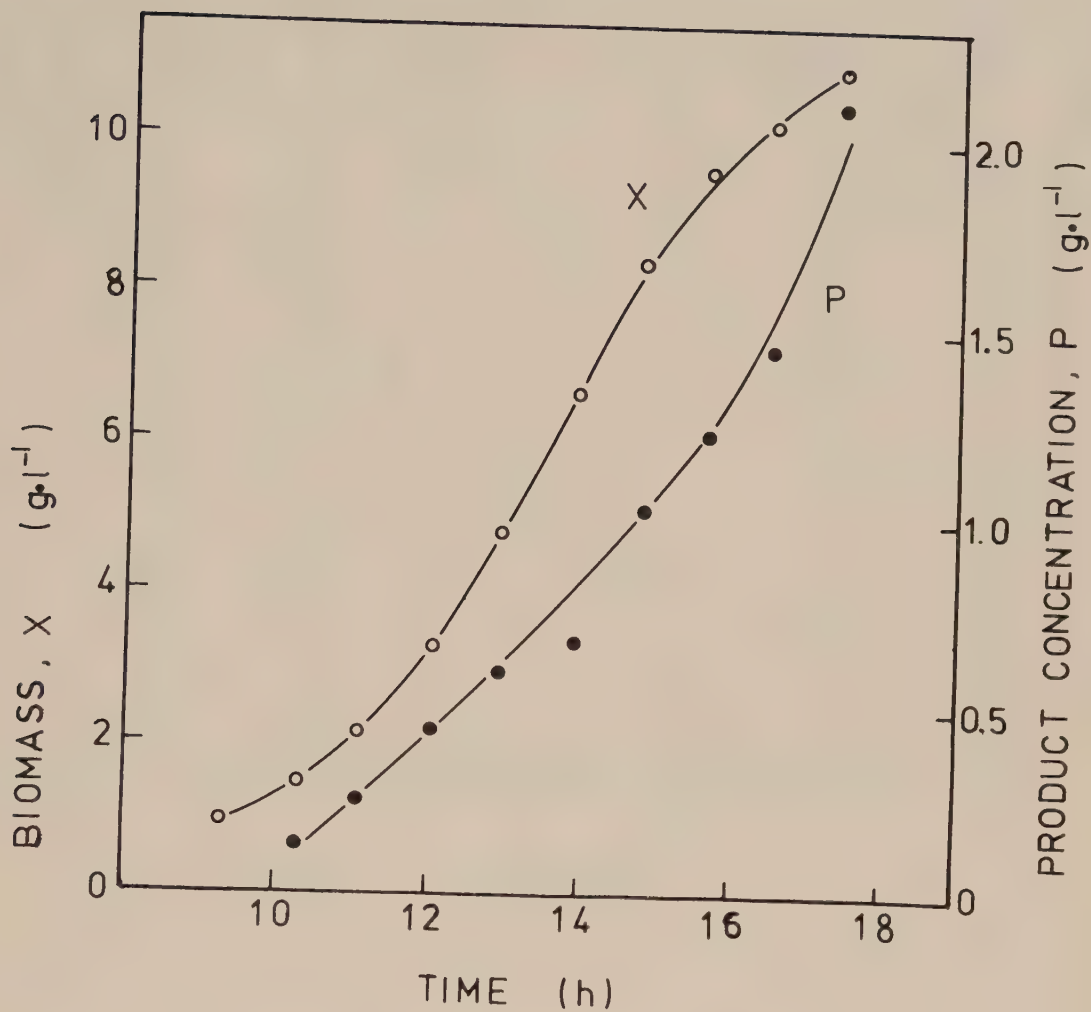


FIG 3. PRODUCTION OF BIOMASS AND AN EXTRACELLULAR POLYSACCHARIDE (GLUCOSE EQUIVALENTS) IN BATCH CULTURE OF STRAIN M13V AT 35°C.

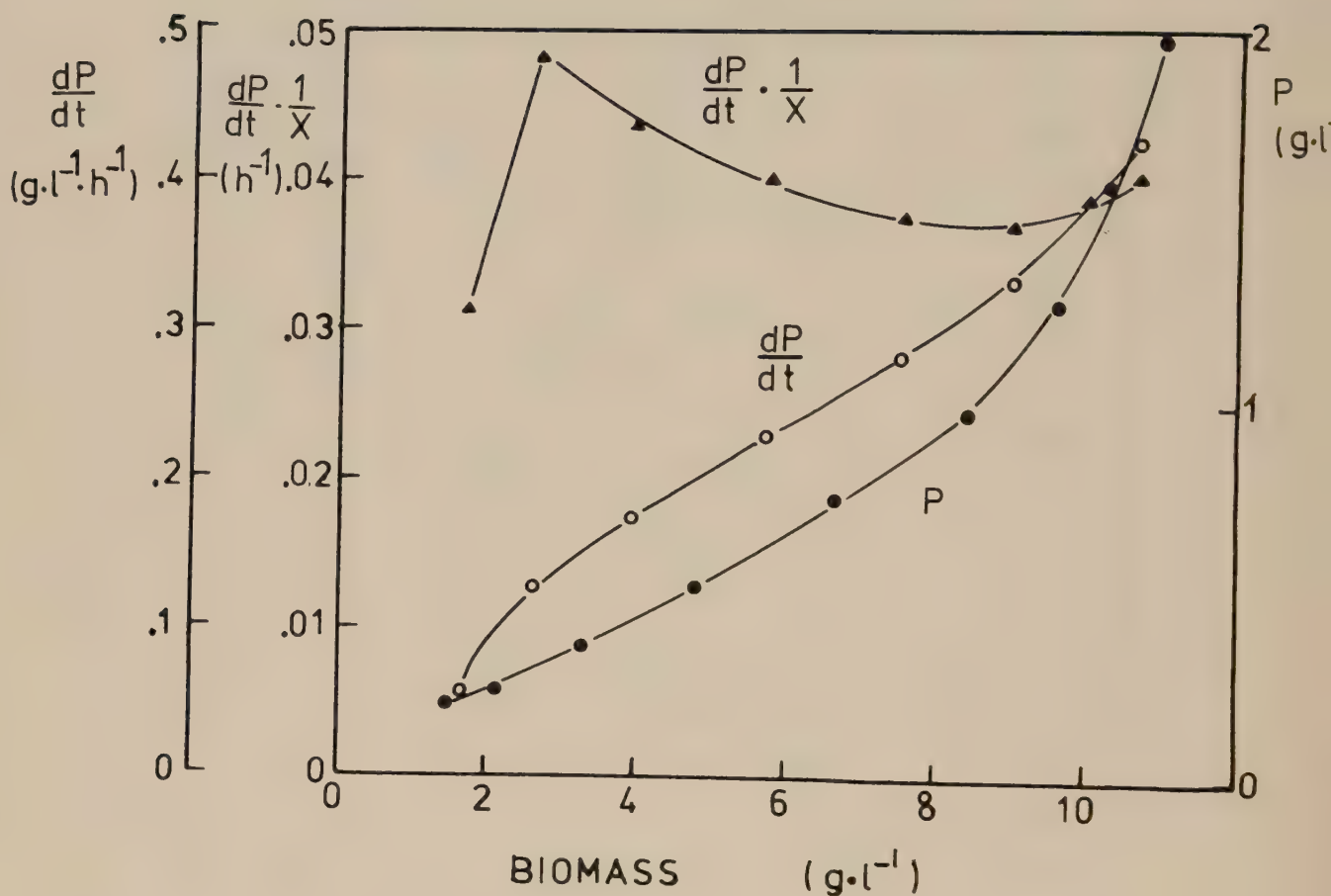


FIG 4. PRODUCTION OF AN EXTRACELLULAR POLYSACCHARIDE (GLUCOSE EQUIVALENTS) IN BATCH CULTURE OF STRAIN M13V AT 35°C. THE PRODUCT CONCENTRATION (P), THE PRODUCTION RATE ($\frac{DP}{DT}$), AND THE SPECIFIC RATE OF PRODUCT FORMATION ($\frac{DP}{DT} \cdot \frac{1}{X}$) ARE SHOWN AS A FUNCTION OF BIOMASS (X).

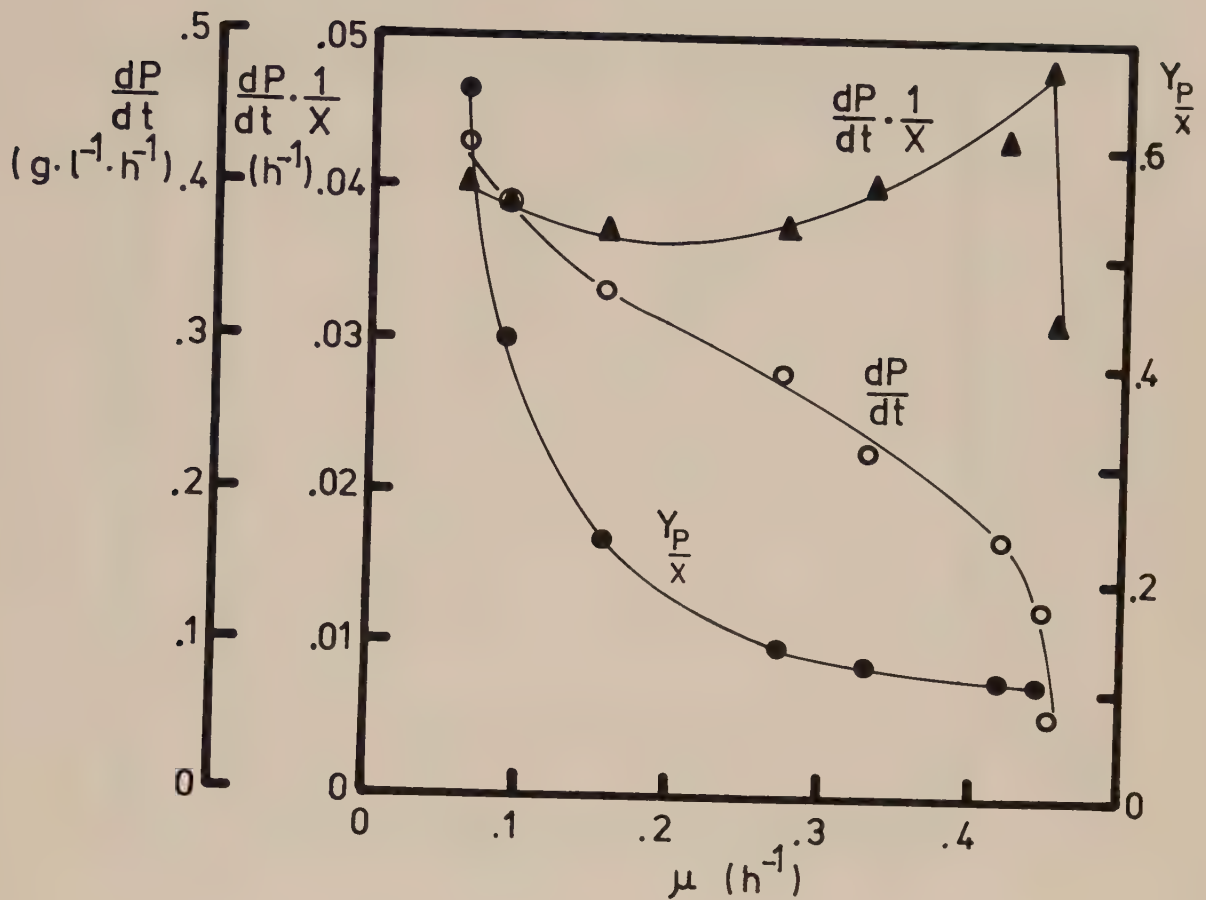


FIG 5. PRODUCTION OF AN EXTRACELLULAR POLYSACCHARIDE (GLUCOSE EQUIVALENTS) IN BATCH CULTURE OF STRAIN M13V AT 35°C. THE SPECIFIC RATE OF PRODUCT FORMATION ($\frac{DP}{DT} \cdot \frac{1}{X}$), THE PRODUCTION RATE ($\frac{DP}{DT}$), AND THE PRODUCT YIELD ($Y_{\frac{P}{X}}$) ARE SHOWN AS A FUNCTION OF THE GROWTH RATE.

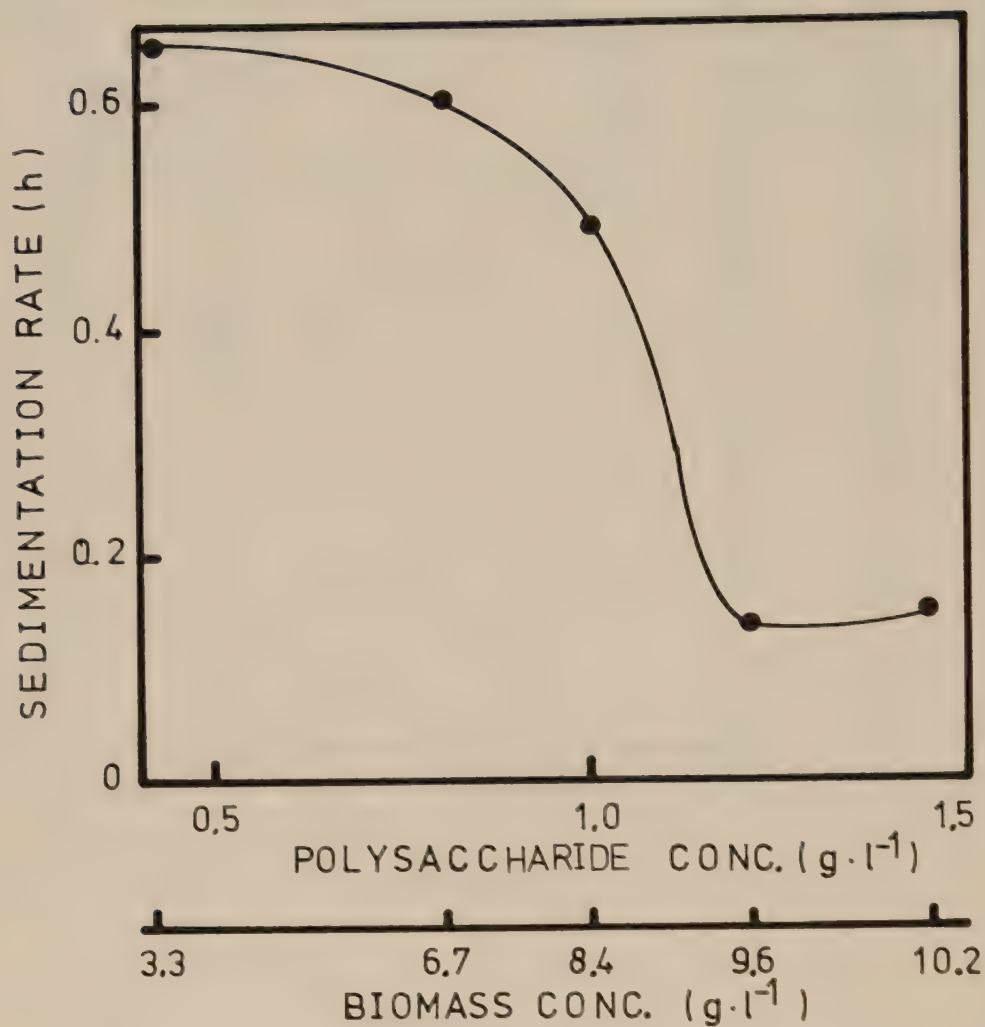


FIG 6. SEDIMENTATION RATE OF STRAIN M13V AS A FUNCTION OF POLYSACCHARIDE CONCENTRATION. THE SEDIMENTATION RATE IS MEASURED AS THE TIME REQUIRED FOR OPTICAL DENSITY TO DECREASE FROM 1.3 TO 0.7 (SEE MATERIALS AND METHODS).

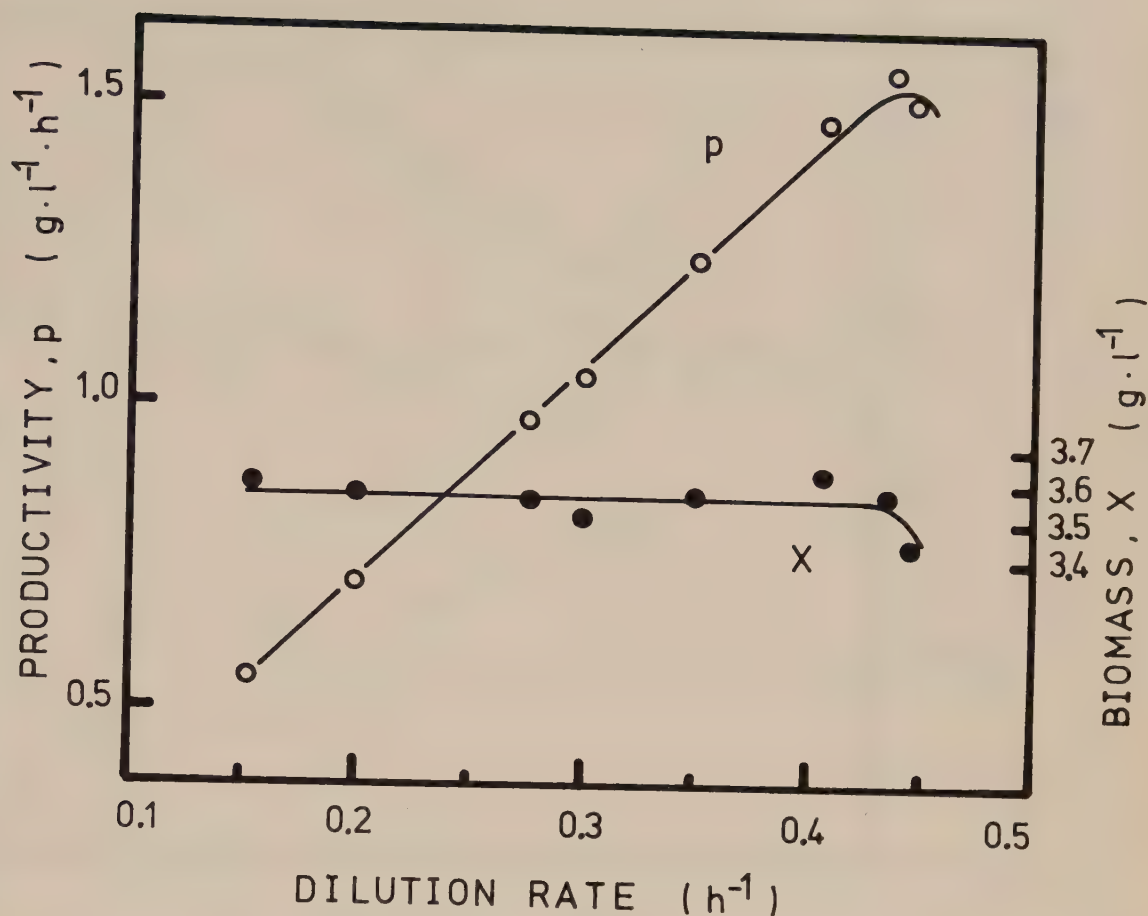


FIG 7. BIOMASS CONCENTRATION AND PRODUCTIVITY OF STRAIN M13V IN A METHANOL LIMITED CHEMOSTAT AT $35^{\circ}C$. METHANOL CONCENTRATION IN INFLOW MEDIUM WAS $11.5 g \cdot l^{-1}$.

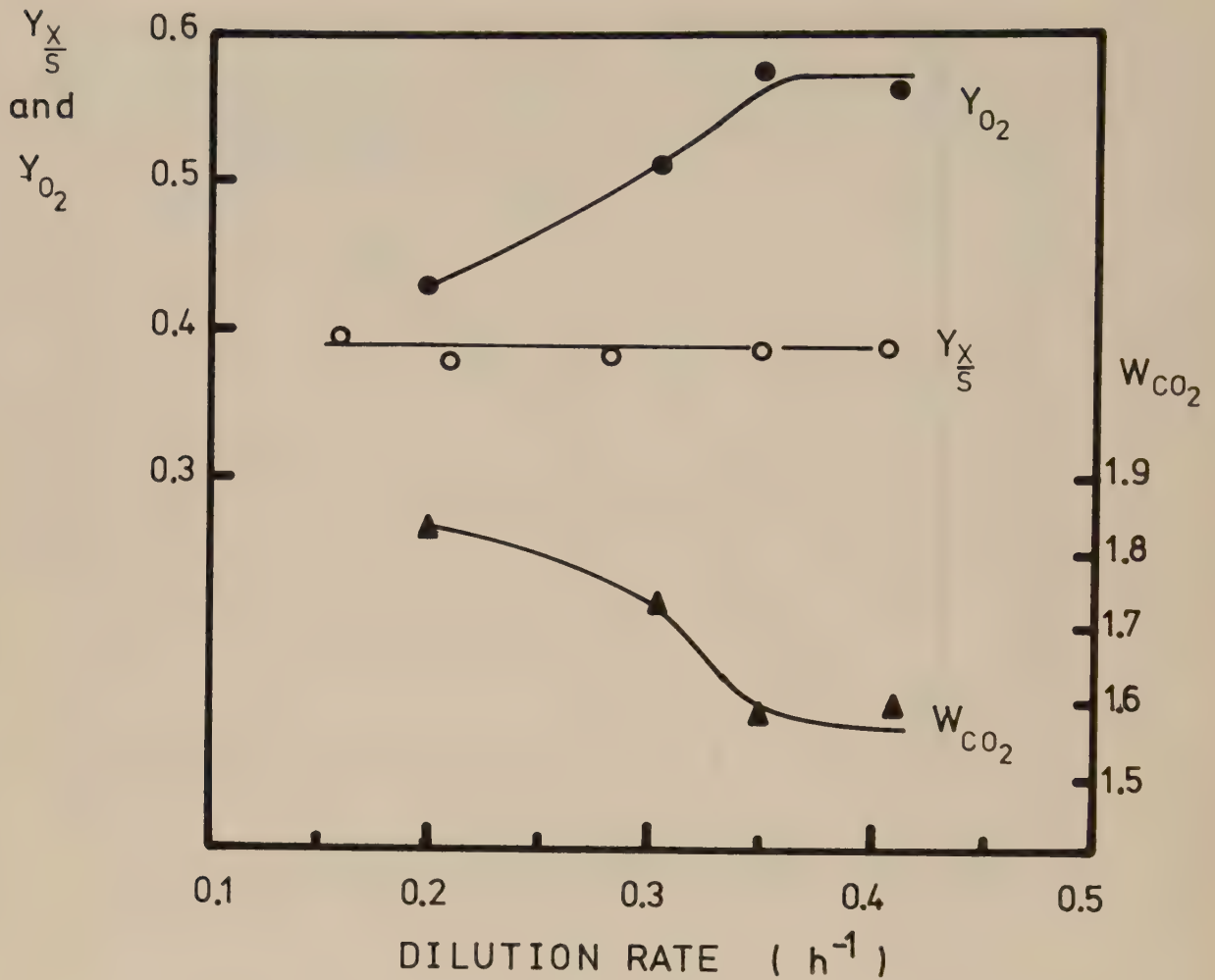


FIG 8. YIELD COEFFICIENT FOR METHANOL (Y_X), YIELD COEFFICIENT FOR OXYGEN (Y_{O_2}), AND^S PRODUCTION COEFFICIENT FOR CARBON DIOXIDE (W_{CO_2}) FOR STRAIN M13V IN A METHANOL LIMITED CHEMOSTAT AT 35°C. METHANOL CONCENTRATION IN INFLOW MEDIUM WAS 4.0 G·L⁻¹ AND BIOMASS CONCENTRATION 1.6 G·L⁻¹.

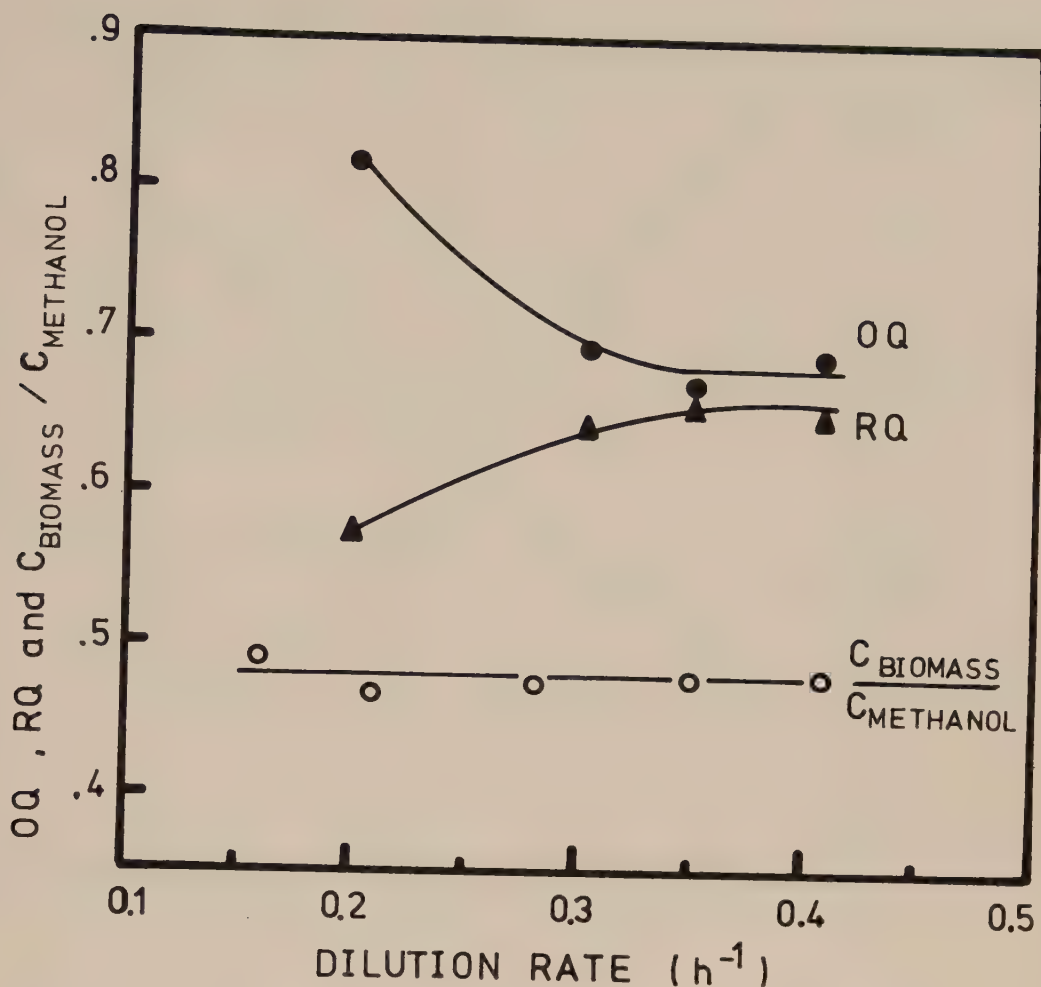


Fig 9. CONVERSION FACTOR FOR METHANOL CARBON ($C_{BIOMASS}/C_{METHANOL}$), OXIDATION QUOTIENT (OQ), AND RESPIRATORY QUOTIENT (RQ) FOR STRAIN M13V IN A METHANOL LIMITED CHEMOSTAT AT 35°C. METHANOL CONCENTRATION IN INFLOW MEDIUM WAS 4.0 G·L⁻¹ AND BIOMASS CONCENTRATION 1.6 G·L⁻¹.

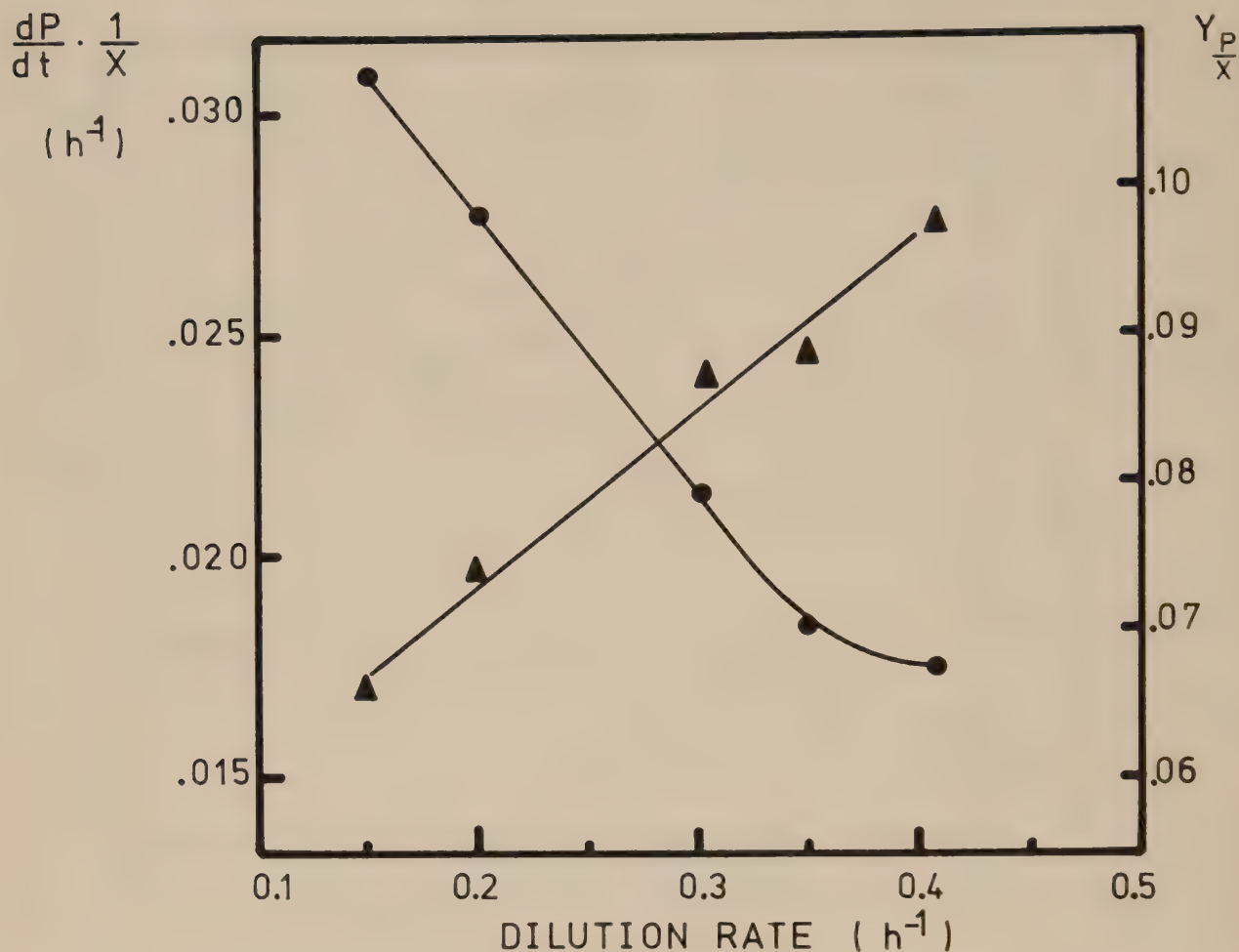


FIG 10. POLYSACCHARIDE PRODUCTION (GLUCOSE EQUIVALENTS) IN STRAIN M13V IN A METHANOL LIMITED CHEMOSTAT AT 35°C. THE PRODUCT YIELD (Y_P/X) AND THE SPECIFIC RATE OF PRODUCT FORMATION ($\frac{dP}{dt} \cdot \frac{1}{X}$) ARE SHOWN AS A FUNCTION OF DILUTION RATE. METHANOL CONCENTRATION IN INFLOW MEDIUM WAS 4.0 G·L⁻¹ AND BIO-MASS CONCENTRATION 1.6 G·L⁻¹.

CONCLUSIONS

1. The mixed methanol utilizing culture TM 20 is an acceptable source for production of SCP, if defined by batch culture experiments. It did not fulfil the requirements when cultivated in continuous culture.
2. A new methanol utilizing bacterium, capable of growth in a mineral salts medium with methanol as the sole source of carbon and energy, was isolated from nature. The organism is an obligate methylotroph and is preliminary classified as Methylomonas methanolica.
3. Methylomonas methanolica holds promise as a suitable strain for SCP production. It has a high growth rate and grows at conditions that correspond to high productivity. The organism utilizes the substrate efficiently and has a high protein content, rich in essential amino acids.
4. A substantial increase in biomass production from Methylomonas methanolica was obtained by using a specially composed mineral medium.
5. A mutant strain, M13V, which had acquired a higher temperature optimum for growth, was obtained from Methylomonas methanolica by means of mutagenic treatment.
6. Strain M13V produces an extracellular polysaccharide. This substance acts as a flocculant, causing the sedimentation of both strain M13V and Methylomonas methanolica.
7. Strain M13V is also attractive as a production strain for SCP. It has the same advantages as the wild strain, and in addition, a higher temperature optimum for growth and the property sediment spontaneously. Productivity values for strain M13V was though lower than for Methylomonas methanolica.

